CHARACTERIZATION OF CYTOSKELETAL PROTEINS FROM IRREVERSIBLE SICKLED CELLS

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CARL C. FLOYD

DEPARTMENT OF BIOLOGY

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CHAPTER I

INTRODUCTION

Irreversible sickled cells, commonly referred to as ISC's, are red blood cells found in individuals with a hereditary hemoglobin disorder called sickle cell anemia. The ISC is best characterized and identified by its sickled shape; a unique property of this cell is its inability to assume the normal biconcave shape. Reasons for this irreversible shape change remain elusive, however, there is compelling evidence that the defect which is responsible for the maintenance of this abnormal shape resides within the cytoskeleton (Lux et al., 1976), a complex of proteins located on the cytoplasmic face of the membrane whose putative functions include the control of: (1) cell shape (Palek and Liu, 1979); (2) membrane integrity (Fairbanks, 1980; Marchesi et al., 1969); (3) mobility of surface determinants (Fairbanks et al., 1978; Sheetz et al., 1980); and (4) deformability (Evans et al., 1977; LaCelle et al., 1977).

Research evidence has revealed only one mutation associated with sickle cell anemia which is in the β-chain of hemoglobin, where glutamic acid is substituted by valine at the sixth position (Ingram, 1956). This defective hemoglobin (hb-S) polymerizes under low 0_2 tension, and as a consequence the cell sickles. It is known that ISC's have an abnormal membrane and a host of other abnormalities, for example, an abnormal distribution of cations (i.e., K^+ and Ca^{+2}) with subsequent cell dehydration. These latter changes may also be due to changes within the membrane. It is speculated that the membrane abnormality occurs when polymerized hemoglobin perturbs the normal state of the membrane.

A correlation between the percentage of ISC's and the severity of the disease has yet to be demonstrated, however, experimental results on rats injected with ISC's revealed that these cells were preferentially lodged in capillaries. If, in fact,

these cells initiate vaso-occlusions in sickle cell anemics, it is conceivable that preventing the formation of ISC's or converting these cells to the normal biconcave shape, should reduce the frequency of vaso-occlusive crisis. Understanding the defect(s) responsible for the maintenance of the sickle shape will possibly lead to the development of new therapy for sickle cell disease.

In a previous attempt to identify the defect(s) responsible for this abnormal shape, cytoskeletons from normal and irreversible sickled cells were compared for protein compositional difference(s). But, no differences were observed (Lux et al., 1976). Dissolution of ISC cytoskeletons in the presence of the detergent sodium dodecyl sulfate (SDS) implies the absence of covalent association between the constituent polypeptides. The interactions responsible for fixation of the abnormal cell shape are presumably non-convalent ones.

Employing electrophoretic techniques of greater resolving power than those previously used, cytoskeletons of normal erythrocytes and ISC's were examined. Furthermore, to identify possible organizational differences between the two cytoskeletons, a battery of crosslinking reagents was utilized.

CHAPTER II

REVIEW OF LITERATURE

Sickle cell anemia is a hereditary disease that is characterized by the production of abnormal hemoglobin. The abnormal molecule called hemoglobin S, was first detected by Pauling et al. (1949), although the nature of the defect in the molecule was not identified until later (Ingram, 1956). The abnormal hemoglobin results from a single point mutation that gives rise to a single amino acid substitution whereby the amino acid valine is substituted for glutamic acid at the sixth position of each beta chain of adult hemoglobin. The amino acid substitution leads to the crystallization of hemoglobin when the molecule is in the deoxyconformation (Bertles and Milner, 1968). As a consequence, the cell shape is distorted into an abnormal sickle shape.

Beet (1949) and Neel (1949) demonstrated that sickle cell anemia was a hereditary disease. They showed that the disease was inherited as if it were due to a single recessive gene. The normal allele is denoted A and the mutated allele which produces abnormal hemoglobin as S. Individuals with the abnormal allele (sickle gene) may have one of the following genotypes, A/S or S/S. Individuals with the S/S genotype have sickle cell anemia, while individuals denoted A/S are heterozygous for the mutant gene and are free of the characteristic symptoms of the disease.

Sickle cell anemics experience a broad spectrum of clinical problems. The most prevalent symptom is the vaso-occlusive crisis, sometimes referred to as the "pain crisis". The pain crisis results from local intravascular sickling, stasis and vaso-occlusion. The severity and frequency of the pain crises are variable. Other acute vaso-occlusive episodes include pulmonary or central nervous system infarction. Another common characteristic among sickle cell anemics is bacterial

infection.

An effective therapeutic treatment for sickle cell anemia is still being sought. In combating the pain crisis, several approaches have been attempted. The current approaches are designed basically to alleviate pain and to protect the affected organs. The primary treatment generally includes analgesics for pain relief, hydration (increased plasma water content to decrease blood viscosity) and antibiotics for bacterial infections. Blood transfusion is generally recommended when an acute crisis is deteriorating; however, transfusions are not recommended for prolonged periods of time, because upon destruction of red cells, "iron overload" may occur, which leads to other clinical problems. Transfusion also causes other problems such as hepatitis and allergic responses.

Murayama (1966) proposed that polymerization of deoxygenated (deoxy) hemoglobin S was due mainly to hydrophobic interactions of the molecule. This concept stimulated the study of chaotropic agents as antisickling agents (Power, 1975). Urea, among the first chaotropic agents to be used was shown to inhibit sickling in vitro (Cerami and Manning, 1971) but was ineffective in vivo at tolerable dosages (Rhodes et al., 1974). Other chaotropic agents have been studied including alkyl ureas, organic solvents and detergents. Although these latter reagents prevent sickling in vitro, they are not without toxic side effects.

Cyanate, a derivative of urea, has been extensively investigated as an antisickling agent. This compound prevents aggregation of hemoglobin by stabilizing the oxygenated form of the molecule (Cerami, 1974). Pilot studies using oral cyanate revealed increased red cell survival, a slight increase in oxygen affinity and a slight decrease in the number of vaso-occlusive crises (Gillette et al., 1974). However, another study demonstrated no decrease in the number of pain crises (Harkness and Roth, 1975) and revealed evidence of toxicity from oral administra-

tion (Harkness and Roth, 1975; Peterson et al., 1974). The administration of cyanate extracorporally is still being investigated as a possible treatment for the disease.

Another therapeutic approach aims at preventing polymerization of hemoglobin S by decreasing the concentration of hemoglobin. Studies by Eaton et al. (1973) and Hofrichter et al. (1974) showed that a small decrease in the concentration of deoxy hemoglobin S can produce a substantial increase in the delay time for polymerization.

Another method of decreasing the concentration of hemoglobin S is to induce the bone marrow to produce either: (1) fetal hemoglobin (Hb-F) or (2) normal hemoglobin (Hb-A) (from newly inserted genes). This represents a potential method for the future.

A third therapeutic approach for combating the painful crisis of sickle cell anemia employs agents that act on the erythrocyte membrane. Cetiedil (a local anesthetic used for the treatment of chronic cardiovascular diseases), is a lipophilic molecule which inserts into the erythrocyte membrane inhibiting sickling of the abnormal cell. The exact mechanism by which it inhibits sickling is unknown. Preliminary studies suggest that the drug alleviates sickle cell crisis. Other agents that act on the erythrocyte membrane are zinc and thorazine (a tranquilizer) both of which are inhibitory of calmodulin, an intracellular Ca^{+2} receptor. Procaine hydrochloride, a cationic anesthetic, has also been demonstrated to decrease membrane rigidity.

Vasodilators have been used for the treatment of pain crisis. Dilation of blood vessels may inhibit entrapment of sickle cells in the microvascular system.

Therapeutic agents discussed here are all effective to some degree, but are not without adverse side effects. Though these agents offer some promise in combating the disease, others are currently under extensive investigation.

In the circulatory system of sickle cell anemics, there exists a population of cells that becomes irreversibly locked into a sickle shape upon deoxygenation. Unlike non-irreversible sickled cells (non-ISC's), irreversibly sickled cells (ISC's) do not return to the normal biconcave shape upon oxygenation. ISC's are abnormal crescent shaped red blood cells (Diggs and Bibb, 1939; Murphy and Shapiro, 1945). In contrast to the normal erythrocyte, ISC's also possess the following abnormal characteristics: decreased viscoelasticity (Chien et al., 1970), increased calcium concentration (Eaton et al., 1973), decreased ATP level (Weed and Bessis, 1975; Sergeant et al., 1969), increased rate of hemolysis in vivo (Bertles and Milner, 1968), decreased K^+ and cell water, increased cell Na^+ (Segal et al., 1974; Glader et al., 1974), increased hemoglobin concentration (Bertles and Milner, 1968), increased membrane associated hemoglobin (Lessin and Wallas, 1973), a faster rate of loss of deformability upon deoxygenation and a slower rate of regain of deformability upon reoxygenation (Hahn et al., 1976).

ISC's vary in proportion in the circulatory system of sickle cell anemics from an average of 5 to 50% (Diggs, 1956; Bertles and Milner, 1968; Sergeant et al., 1969). It has been proposed that ISC's contribute to pain crisis, yet there is no correlation between the percentage of ISC's and the frequency at which the pain crisis occurs (Diggs, 1956). There is a direct correlation between the percentage of ISC's and the rate of hemolysis (Sergeant et al., 1969; McCurdy and Sherman, 1978), the malfunction of the spleen (Sergeant, 1970) and conjunctival blood vessels abnormalities (Sergeant et al., 1972). In a rat model, ISC's are preferentially lodged in capillaries, suggesting that they may indeed play a roll in initiating vaso-occlusive crisis (Klug et al., 1974).

The exact mechanisum by which ISC's are formed is not known. There are many factors which seem to contribute to their formation. It is possible that the

most critical alteration is membrane damage, which may result from repeated cycles of sickling and unsickling (Padilla et al., 1973).

Shen et al. (1949) demonstrated that prolonged anaerobic (without oxygen) incubation leads to the formation of ISC's. Other factors possibly leading to ISC formation include: decreased intracellular K^+ (Glader and Miller, 1975); increased Ca^{2+} concentration (Eaton et al., 1973); membrane loss (Bertles and Milner, 1968); and membrane-associated hemoglobin (Lessin and Jensen, 1974). The available data suggests that all of these abnormal properties, in conjunction with each other contributes to the formation of ISC's.

In red blood cells, the structural components for shape appear to be contained within the membrane since isolated membranes retain the biconcave shape characteristic of the intact cell. Beneath the lipid-protein bilayer exists a network of peripheral membrane proteins called the cytoskeleton. The cytoskeleton is defined operationally as the interconnected proteins that remain after a cell or membrane (ghost) has been extracted with a non-ionic detergent such as Triton X-100.

Electron microscopic studies by Nicholson et al. (1971) showed that the cytoskeleton is closely associated with the cytoplasmic surface of the membrane. Yu et al. (1973) were the first to demonstrate that the cytoskeleton retained the approximate size and shape of the intact red cell. The appearance of the erythrocyte cytoskeleton has been studied by Hainfield and Steck (1977), Sheetz (1979) and described by Tilney and Detmers (1975) as "...an anastomosing framework like a net woven by a myopic fisherman."

The cytoskeleton comprises approximately 60% of the total membrane protein mass and includes the following: Spectrin (bands 1 and 2), Actin (band 5), bands 4.1, 4.9 and 7 (Sheetz, 1979). Other proteins found associated with the cytoskeleton are

Ankyrin (bands, 2.1, 2.2, 2.3 and 2.6), bands 3 and 4.2. These latter proteins are not considered to be integral components of the cytoskeleton. Band 3 appears to be the major anion channel in the membrane (Rothstein et al., 1979) and ankyrin attaches the cytoskeletal network to the membrane via the cytoplasmic domain of band 3. The function of band 4.2 has not yet been defined.

Spectrin is the major protein of the network and comprises about 75% of the cytoskeleton protein mass (Lux et al., 1976). It is composed of two subunits, band 1 (α-chain, molecular weight - 240,000 daltons) and band 2 (β-chain, molecular weight - 200,000 daltons) which are structurally similar but nonidentical (Anderson and Tyler, 1979). Spectrin exists as a heterodimer (1+2) or heterotetramer (1_2+2_2) (Ralston et al., 1977). The tetramer is thought to be the physiological species in the erythrocyte (Ungelwickell and Gratzer, 1978).

Several investigators have suggested that erythrocyte actin (molecular weight = 55,000 daltons), is in a nonfilamentous state (i.e., G-actin), complexed with other cytoskeletal proteins (Pinder et al., 1975; Tilney and Detmers, 1975). This idea remains a controversial issue. Actin filaments are not visible in electron micrographs of red blood cell membranes and the amount of actin present is not compatible with the existence of long actin filaments, however, short oligomers may be present.

Studies of band 4.1 (molecular weight = 78,000 daltons) indicate that it plays an integral role in maintaining the supramolecular structure of the cytoskeletal complex. Fowler and Taylor (1980) reported that an oligomeric complex of spectin, actin and band 4.1 causes actin to gel and that purified spectrin and actin tetramers lack this activity, suggesting that band 4.1 is required for crosslinking. Tyler et al. (1980) reported that purified band 4.1 binds to spectrin in the absence of actin indicating a specific interaction of 4.1 with spectrin.

An important function of the cytoskeleton is the control of red cell shape. Molecules such as ATP and 2,3-Diphosphoglycerate have been shown to dissociate the cytoskeletal complex (Sheetz et al., 1980). It has also been demonstrated that these metabolites cause a shape transformation from a discocyte to an echinocytic shape and concommitant vesiculation. Cytoskeletons extracted from ISC's retain the shape typical of the ISC suggesting that defects responsible for the abnormal shape resides within this complex (Lux et al., 1976); the defect(s) itself has yet to be identified.

In some hemoglobinopathic cells, the defects within the cytoskeleton are better understood. Cytoskeletons from hereditary pyropoikilocytes (HPP) are unstable and exhibit irregularities in electron density, suggesting abnormalities in cytoskeletal assembly (Palek, 1981). HPP spectrin exhibits abnormalities which include decreased extractability from membranes (Wiley and Gill, 1976) and increased susceptibility to thermal denaturation (Chang et al., 1979). Cytoskeletons from hereditary elliptocytes (HE) also exhibit an abnormal shape. In these red cells, spectrin is abnormally heat sensitive and denatures at a lower temperature than normal (Tomaselli et al., 1981). Some patients with hereditary elliptocytosis have spectrin that yields abnormal trypsin-digestive fragments (Coltzer and Zail, 1981) and cytoskeletons from some patients with elliptocytosis have been observed to lack band 4.1 (Cohen and Branton, 1981; Tchernia et al., 1981). Studies on hereditary spherocyte (HS) cytoskeletons suggest that interactions between band 4.1 and spectrin in some patients is defective (Cohen and Branton, 1981). A mutant strain of the mouse Mus musculus exhibits a severe form of spherocytic anemia. Erythrocyte membranes from this mutant show a marked decrease in spectrin (Greenquist et al., 1978). Spectrin deficiency correlates with the degree of hemolysis (Lux, 1979) and incorporation of normal spectrin into these membranes results in improved

structural integrity (Shohet, 1979). These findings suggest that defects within the cytoskeleton may be responsible for the abnormal cell shapes. To say that they are the primary lesions of the cells rather than secondary ones remains unanswered.

The cytoskeletal complex also participates in the stability of the membrane, its deformability and the lateral mobility of certain transmembrane proteins (Lux, 1979; Palek and Liu, 1979).

CHAPTER III

MATERIALS AND METHODS

SOURCE OF BLOOD

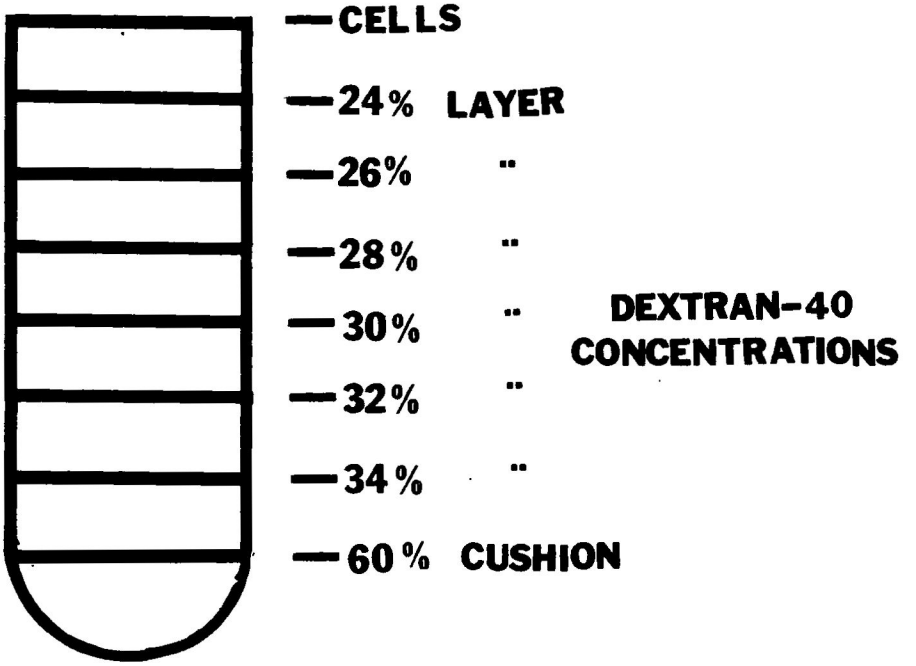
Blood was obtained from the Sickle Cell Foundation of Georgia and Herman Harris, Administrative Officer of the Comprehensive Sickle Cell Center of the Medical College of Georgia, Augusta, Georgia. Blood obtained had been identified as homozygous for the sickle (S/S) gene. Individuals had not been transfused within a three month period prior to phlebotomy. Normal (A/A) blood was obtained from paid volunteers.

SEPARATION OF IRREVERSIBLE SICKLED CELLS (ISC's)

Blood was transferred to clinical centrifuge tubes and centrifuged for ten minutes (min) in a Dynac Clinical centrifuge at 100 rpm at $4^{o}C$. The plasma and buffy coat were removed and discarded. The remaining packed cells were suspended in wash buffer called 5PN(8) (0.15M NaCl, 5mM $NaPO_4$, pH 8), centrifuged as above and the supernate discarded. The procedure of Abraham et al. (1975) was adopted for separating ISC's with some modifications. Dextran - 40 solutions of various concentrations were prepared by dissolving appropriate quantities in a suspension medium consisting of 585 mg KH_2PO_4, 3141 mg $NaH_2PO_4.H_2O$, 8600 mg glucose, 6277 mg NaCL, 96 mg $MgSO_4$ with water added to make the total volume 1000 ml (pH 7.4). A blood volume of 1.5 to 2.0 milliliters (ml) was layered on a Dextran-40 (molecular weight 40,000) discontinuous gradient ranging from 24% to 34% with a 60% cushion to prevent pelleting of cells (Fig. 1). The total volume was 34 ml with 4.5 ml for each layer. Blood was centrifuged in a Beckman 25.1 swinging bucket rotor in a Beckman L5-50 Ultracentrifuge at a speed of 90,000 x g (24,000 rpm) at $4^{o}C$ for 1.5 hours (hr). Centrifugation of cells (normal and sickle) resulted in the

Figure 1. Diagrammatic representation of the Dextran - 40 step gradient used to fractionate erythrocytes. Each layer consisted of 4.5 ml of the appropriate concentration of Dextran - 40.

DEXTRAN DENSITY GRADIENT



34 milliliters total capacity

separation of six distinct bands of red blood cells. The bottom band in the sickle gradient appeared darker in intensity than the normal bottom band. After centrifugation, the two closest fractions were combined to yield three fractions varying in the percentage of ISC's: Top fraction (approximately 9%), middle fraction (approximately 20%) and the bottom fraction (approximately 71%). Fractions were washed twice in 5PN(8) and subsequently centrifuged in an International Equipment Centrifuge (IEC) for ten min at 35,000 x g (16,000 rpm) and the supernate was discarded.

SCORING OF ISC'S

Packed cells were resuspended in 5PN(8) and incubated under 95% oxygen and 5% carbon dioxide for five min with mild agitation. An aliquot of suspended blood was removed and fixed in 4% glutaraldehyde in 5PN(8) solution. Cells from each fraction were viewed with a Swift International Phase Contrast Microscope. ISC's were defined as elongated crescent shaped cells whose length to width ratio was greater than 2:1. A total of 500 cells were counted to determine the percentage of ISC's in each fraction. Normal cells were also viewed with the phase contrast microscope to determine if the fractionating conditions altered the cell morphology.

PREPARATION OF MEMBRANES AND CYTOSKELETAL COMPLEXES

The method of Fairbanks et al. (1971) was employed for preparing membranes (Fig. 2). Packed intact cells were washed three to four times with an isotonic buffer (5PN(8)) in a clinical centrifuge and then lysed in a hypotonic solution called 5P(8) (5mM sodium phosphate, pH 8.0). They were subsequently washed three to four times in the same buffer using the IEC centrifuge until membranes were free of hemoglobin (white).

Cytoskeletal complexes from both intact cells and membranes were prepared according to the procedure of Sheetz (1979) with the following modifications (Fig.

Figure 2. General scheme for analyzing membrane proteins.

3). Cells or membranes (ghosts) were suspended 1:1 in extraction buffer (6M KCl, 48mM Hepes/Ringer (pH 7.4), 1.0mM $MgCl_2$, 0.05mM $CaCl_2$ and 3% Triton X-100). The suspended cells (or membranes) were extracted for 30 min at 4°C (in an ice bath) at 120 oscillations per min on a rotary shaker. Samples were then centrifuged for 30 min in the IEC at 65,000 x g at 4°C. The supernate was aspirated and discarded; the pellet (cytoskeletal complexes) was resuspended in 5PN(8) and washed until free of hemoglobin. The washed pellet was resuspended in 5PN(8) at a concentration of three to four milligrams (mg) per ml. The cytoskeletal protein concentration was assayed by the Lowry (1951) method.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

One dimensional polyacrylamide gel electrophoresis was performed as described by Fairbanks et al. (1971) and Laemmli (1970). Four percent cylindrical (5mm i.d. x 10.5 cm) Fairbanks gels were prepared from a stock of 30% acrylamide and 0.8% bisacrylamide. The Laemmli system was used to prepare slab gels.

Polyacrylamide slab gels were prepared in the Hoeffer Vertical Slab Apparatus, with glass plate dimensions of 1.5 millimeters (mm) x 14 centimeters (cm) x 18 cm. After the plates were assembled, 5% and 15% acrylamide solutions were prepared, each of which had a total volume of 12.5 ml. Twelve microliters (µl) of tetramethylethylenediamine (TEMED) and 24 µl of 10% ammonium persulfate (catalysts) were added to the 15% solution and the solution was then poured into the exit chamber of a linear gradient maker. The valve connecting the two chambers was briefly opened to allow back flow of the 15% solution to prevent entrapment of bubbles in the canal between the two chambers. The 5% acrylamide solution was subsequently added to the other chamber of the gradient apparatus, after adding 12 µl of TEMED and 32 µl of 10% ammonium persulfate. Stirring bars were added to each chamber, and the exit port and valve between chambers opened. The

Figure 3. General scheme for preparing cytoskeletons from membranes and intact red blood cells, and analyzing their protein composition.

MEMBRANES OR RED BLOOD CELLS (SICKLE OR NORMAL)

EXTRACT WITH 3% TRITON X-100
IN EXTRACTION BUFFER

MEMBRANE OR RED BLOOD CELL EXTRACT

CENTRIFUGE (40,000 X G)

SUPERNATE
(SOLUBLE FRACTION)
DISCARD

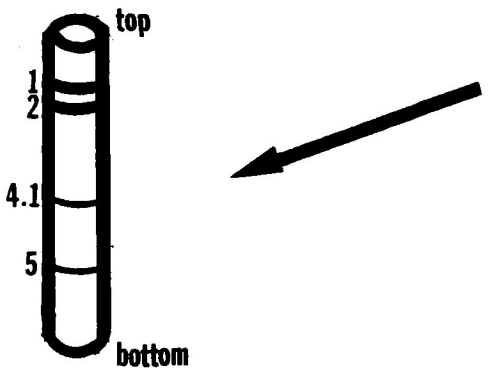
PELLET (INSOLUBLE FRACTION,
CYTOSKELETONS)

SUPERNATE

CENTRIFUGE IN WASH BUFFER
(ISOTONIC 5PN)
UNTIL FREE OF HEMOGLOBIN

PELLET (WASHED CYTOSKELETONS)

SOLUBILIZE, SUBJECT TO
ELECTROPHORESIS, STAIN AND
DESTAIN.



TYPICAL APPEARANCE OF CYTOSKELETAL
PROTEINS ON POLYACRYLAMIDE GELS
AFTER STAINING WITH COOMASSIE
BLUE AND DESTAINING.

acrylamide gradient solutions exiting the gradient maker filled part of the volume between the two plates in the gel apparatus. The acrylamide solution between the two plates was subsequently overlayered with butanol and allowed to polymerize for one hour. After the gel (separating) was polymerized, the butanol overlayer was removed and replaced with Laemmli electrophoresis buffer. The gel was then allowed to stand overnight at room temperature. The overlayer solution was then removed and after blotting the separating gel interface with a soft tissue, a 4.5% stacking gel solution was poured on top. A 10-well comb was inserted into the stacking gel prior to polymerization to form the lanes for the samples to be applied. After 30 min, the comb was carefully removed from the polymerized gel and the residual unpolymerized gel solution aspirated.

The base of the gel chamber was removed and the gel was placed inside the lower buffer reservoir. The upper and lower reservoirs were filled with electrophoresis buffer and the samples applied in the various lanes. A cannular (pipette bent at the end) was used to remove bubbles from the bottom of the glass plates. The electrodes were then connected to a power supply that maintains constant milliamperes (ma). The power supply was adjusted to 20 ma per gel. Electrophoresis was terminated when the tracking dye was at the end of the gel. The gel was then removed from the glass plates by prying them apart with a spatula. It was placed in 0.05% Coomassie blue R-250, 25% isopropyl alcohol, 15% acetic acid (Fairbanks et al., 1975), and stained a minimum of 12 hours. Subsequently, it was destained in 10% isopropyl alcohol and 10% acetic acid. After destaining, the gel was viewed upon a fluorescent lamp box.

Isoelectric Focusing (IEF)

Isoelectric Focusing was performed as described by O'Farrell (1975). Ampholytes were omitted from the solubilization buffer and the urea concentration

in the buffer was adjusted to its saturation point. Gels were poured as described by O'Farrell and overlayed with butanol. After two to three hours, the butanol was replaced with 20 µl of sample overlayer solution and the gel was allowed to polymerize for another one to two hours. After polymerization, gels were mounted in the standard cylindrical gel apparatus. They were prefocused for 15 min at 200 volts, 30 min at 300 volts, and finally 30 min at 400 volts. The upper (cathode) gel chamber was filled with fresh 0.025 M NaOH, which had been degassed by boiling and stored in a tightly sealed container. The lower (anode) reservoir was filled with 0.03M H_2PO_4.

One volume of protein sample at a concentration of 3-4 mg/ml was diluted in 0.5 ml of 2% (w/v) NP-40 and 5% β-mercaptoethanol. Samples were vortexed and boiled for five min and again subjected to mixing. They were then cooled in a cold water bath and crystalline urea was added to a concentration of 9M. Samples were then centrifuged in a Brinkman Micro Centrifuge for two min and the supernate was applied to the first dimension tube gel (IEF) and overlayed with diluted sample overlayer solution.

Electrophoresis was performed at 400 volts for 12 hours and 800 volts for the 13th hour. Proteins were identified by the staining procedure of Tuszynski et al. (1978).

A blank isofocused gel was used to determine the pH gradient when electrophoresis was ended. Gel pH was measured with an automatic single contact electrode, connected to a standard pH meter. In some cases, pH readings were made after cutting a blank gel in 5mm slices and placing each into 2 ml of degassed water, where they were shaken for five to ten min. The pH of each solution was subsequently determined with the pH meter.

Isofocused gels to be subjected to SDS-electrophoresis (second dimension) were

not stained but equilibrated with one volume of 0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol and 5% Beta-mercaptoethanol for one min by mild agitation.

Two-Dimensional Polyacrylamide Gel Electrophoresis (IEF/SDS PAGE)

IEF Gels to be placed on SDS-Slab gels were equilibrated as described above. After equilibration, they were sealed on top of the stacking gel with a 1% agarose solution. Electrophoresis was initiated at 100 volts until the dye front entered the separating gel and then the voltage was increased to 125 volts. Electrophoresis was ended when the tracking dye reached the bottom of the gel. Gels were removed from the vertical apparatus and stained according to Fairbanks et al. (1971).

GELCODE STAINING PROCEDURE

Polyacrylamide slab gels were shaken in 5% ethyl alcohol (ETOH) - 10% acetic acid (HAc) for two hours and this was repeated once in fresh solution. They were then transferred to a 40% ETOH - 10% HAc solution and again stirred for two hours. This latter solution was then replaced with 10% ETOH - 0.5% HAc and the gels shaken for one hour; following this step, they were shaken for another hour in fresh 10% ETOH - 0.5% HAc.

The final fixing solution was decanted and Gelcode reagent 1 (entire contents dissolved in 1000 ml H_2O) poured on to the gel. The gel was then shaken for two hours.

Reducing reagent (2.25 ml of reagent 4 per 300 ml of solution 2 (reagent 2 plus reagent 3 dissolved in 2000 ml)) was prepared fresh and added to the gel after washing the gel for 10-20 seconds in deionized water. The gel was shaken in reducing agent for ten min. Usually color development began immediately upon adding reducing reagent; it was stopped by adding 5% HAc. Gels were subsequently stored in this latter solution.

SILVER STAINING PROCEDURE

Polyacrylamide slab gels were stirred in 40% methanol (MeOH) - 10% HAc for 30 min, and after decanting the above solution, the gels were stirred in 10% ETOH - 5% HAc for 15 min. The latter solution was replaced with a fresh solution and stirring continued for 15 min.

After removing ETOH - HAc, oxidizing solution (BioRad) (20 ml commercial stock dissolved in 180 ml deionized water) was added to the gel. The gel was allowed to stand in this solution for 20 min with occasional shaking. The oxidizing solution was decanted and the gel then washed once with deionized water. The gel was then placed in silver reagent (20 ml stock silver solution and 180 ml deionized water) for 20 min with mild agitation and then washed with deionized water until the gel became clear.

Developing solution (BioRad) (entire contents of commercial stock plus 3.6 liters of deionized water) was added to the gel after removing the water wash. The gel was allowed to stand in developer for 30 seconds with constant agitation, before the solution was replaced with fresh developer. The gel was then allowed to stand for five min in the latter solution with gentle agitation. This last step was repeated until the bands reached the desired intensity. Development was stopped by decanting the developer and adding 5% HAc. Gels were subsequently stored in this latter solution.

CROSSLINKING OF CYTOSKELETAL COMPLEXES

A. O-phenanthroline/Copper Sulfate: (O-phe/$CuSO_4$) dissolved in 5mm Sodium Phosphate buffer, pH 8.0 (5P(8)) was added to 20 volumes of cytoskeleton suspension (i.e., 5 ml O-phe/$CuSO_4$ to 100 ml of cytoskeleton suspension). Cytoskeletons were crosslinked with O-phe/$CuSO_4$ (Steck, 1972) at various concentrations ranging from 1mM to 10mM. Incubations were carried out on a rotatory

shaker at 120 oscillations per min at various time intervals, from 0 to 60 min. The oxidative crosslinking reaction was stopped using 4X Fairbanks solubilizing buffer (4% SDS, 8mM Tris Acetate, 20% sucrose and pyronin y) at one-fourth the volume of the cytoskeletons in the reaction mixture. Dithiothreitol (0.0248 grams (gm) per ml of solubilizing buffer) was added to control cytoskeletons without crosslinking reagent.

B. Glutaraldehyde: Cytoskeletons suspended in 5PN(8) were crosslinked with glutaraldehyde (Steck, 1972). Crosslinking was allowed to occur at room temperature at various concentrations and time periods and then stopped by the addition of 4X Fairbanks solubilizing buffer containing dithiothreitol (0.0248 gm DTT per ml of solubilizing buffer) at one-half the volume of cytoskeletons.

C. Dimethy 3,3'-Dithiobispropionimidate (DTBP): Cytoskeletons were suspended in 0.05M Triethanolamine buffer (pH 8.0) at a final concentration of 2 mg protein per ml. DTBP dissolved in the same buffer was added in a 1:1 ratio and the cytoskeleton suspension was incubated at room temperature at various times and concentrations. Crosslinking was terminated with 50 µl of 1M ammonium acetate (NH_3Ac) per µl of sample. The reaction mixture was then incubated at room temperature for ten min and subsequently 50 µl of N-ethylmaleimide (NEM) (20 mg per ml) and 200 µl of 20% (w/v) SDS were added (Wang and Richards, 1974). One mg/ml of Pyronin y was used as a tracking dye.

D. Dithiobis (succinimidyl propionate) (DTSP): Cytoskeletons were suspended in 0.05M Triethanolamine buffer (pH 8.0) at a final concentration of 4mM DTSP. DTSP dissolved in the same buffer was added in a 1:5 ratio and the cytoskeleton suspension was incubated at room temperature at various times and concentrations. Crosslinking was terminated with 200 µl of 20% (w/v) SDS in 25mM $NaPO_4$, 1mM $MgCl_2$, pH 8, and 1mg/ml of Pyronin y (Brandon, 1980).

E. Dimethyladipimidate (DMA) and Dimethylsuberimidate (DMS): DMA and DMS (60 mM) were dissolved in 0.5M Triethanolamine (pH 9.0) for separate incubations. An aliquot of the crosslinking solution was added to the cytoskeleton suspension in a 1:1 ratio. The reaction mixture was incubated at room temperature at various time periods. Crosslinking was quenched by the addition of 4X Fairbanks buffer at one-half the volume of the cytoskeletons. DTT (0.0248 g/ml of solubilization buffer) was added to the controls and to the experiment sample.

PHOTOGRAPHY

Gels were photographed with a Polaroid 525 land camera with type 52 Polaroid land film. A yellow filter was used to enhance contrast.

SCANNING ELECTRON MICROSCOPY

Red blood cells and membranes were fixed for 12 hrs by adding 1 volume of 4% glutaraldehyde in 5PN(8) to 1 volume of sample. Samples were washed one to two times for 15 min in deionized water, and dehydrated through a graduated series of ethanol. Samples were then air dried or critical point dried using liquid carbon dioxide and subsequently coated with platinum. They were viewed with an ETEC Omniscan Scanning Microscope.

CHAPTER IV

EXPERIMENTAL RESULTS

EVALUATION OF TECHNIQUES FOR PREPARING CYTOSKELETONS

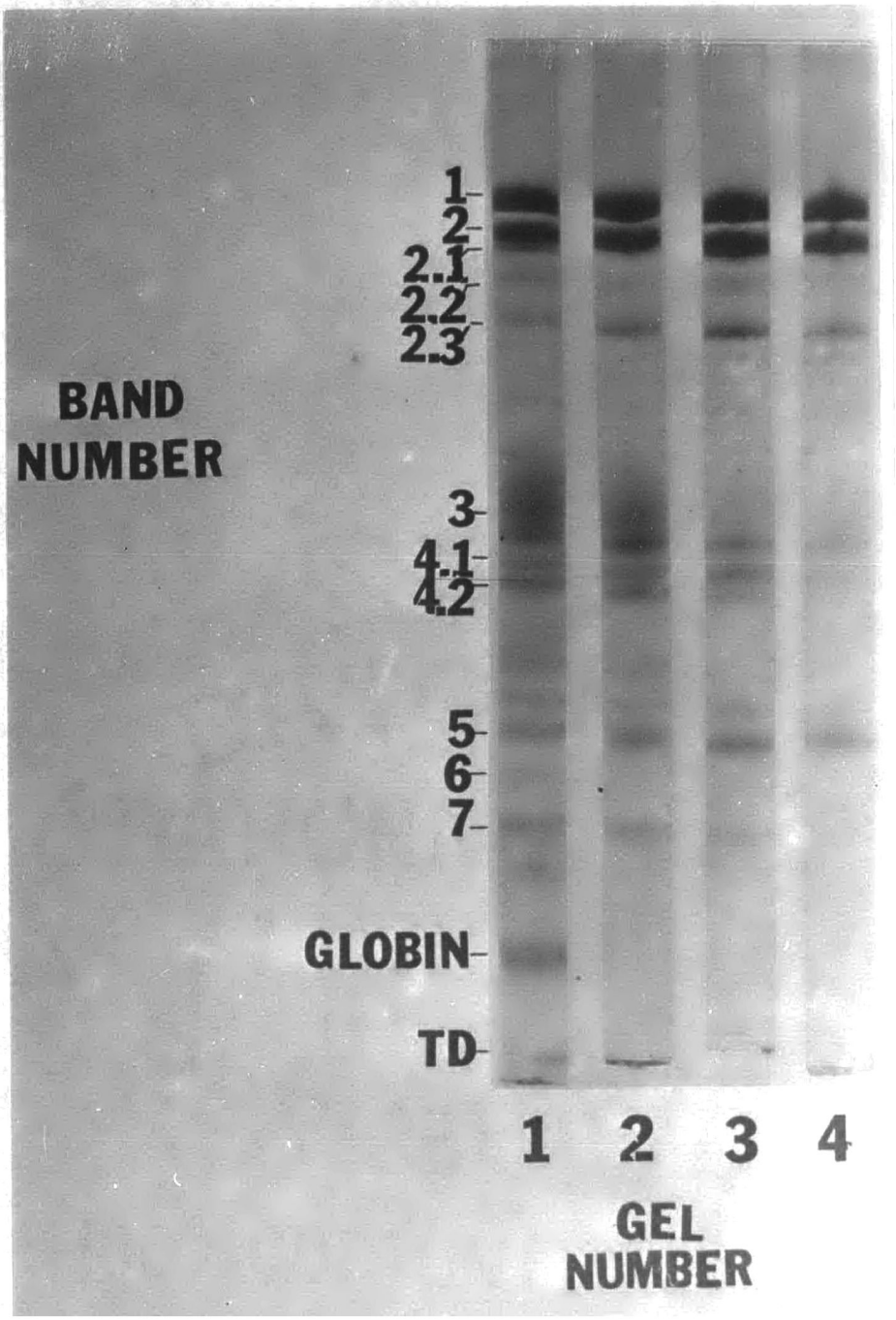
Comparison of Lux and Sheetz Procedures

Several techniques have been utilized to prepare erythrocyte cytoskeletons (Lux et al., 1976; Sheetz, 1979). The following analyses were carried out in order to directly compare these methods as well as to corroborate the value of the different chemicals present in the buffer. A goal of this work was to define a cytoskeleton of minimal protein composition which retained the shape of the cell. However, structural definition of a minimum cytoskeleton has not yet been achieved because of difficulties in visualizing isolated cytoskeletons with the electron microscope.

When membranes were extracted according to Lux et al. (1976) with 0.5% Triton X-100, 56mM NaB_4O_7 (pH 8.0), at 0^oC for 30 min, a cytoskeleton was obtained containing all of the major cytoskeletal components: Spectrin (Bands 1 and 2), band 4.1, band 4.9, band 5 (actin) and small amounts of the non-cytoskeletal polypeptides, bands 2.2, 2.3, 4.2 and 3 (Fig. 4, lane 2). In an attempt to remove all of the non-cytoskeletal proteins, the concentration of the detergent (Triton X-100) was increased ten fold (to 5%). High concentrations of Triton X-100 were effective in removing the majority of band 3 and the other non-cytoskeletal proteins, yet small amounts of the polypeptides remained associated with the cytoskeleton (Fig. 4, lane 3).

Extraction of cytoskeletons employing Sheetz's procedure (1979) was more effective in removing non-cytoskeletal constituents. The extraction buffer is a Hepes-Ringer buffer (pH 7.4) containing 6M KCl and 5% Triton X-100. It has been demonstrated by Sheetz (1979) that KCl augments the effect of Triton in the

Figure 4. Electrophoresis of proteins in Triton - cytoskeletons prepared by the Lux or Sheetz procedure. Electrophoresis was carried out on Fairbanks 4% sodium dodecyl sulfate (SDS) polyacrylamide gels and the proteins visualized after staining with Coomassie brilliant blue. Gels: 1) Whole erythrocyte membranes; 2) Triton - cytoskeletons prepared by the Lux procedure (0.5% Triton X-100); 3) Triton - cytoskeletons prepared by the Lux procedure (5% Triton X-100); 4) Triton - cytoskeletons prepared by the Sheetz procedure (5% Triton X-100). Nomenclature for erythrocyte membrane proteins after Fairbanks et al (1971).


BAND
NUMBER
1
2
2.1
2.2
2.3
3
4.1
4.2
5
6
7
GLOBIN
TD
1
2
3
4
GEL
NUMBER

removal of integral membrane proteins (e.g., band 3). These cytoskeletons were simpler in protein composition in that the majority of the non-cytoskeleton proteins (bands 2.1, 2.2, 3 and 4.2) were absent (Fig. 4, lane 4).

The analyses in the following section were carried out to examine the effect of the various components of the Sheetz extraction buffer on the cytoskeleton protein composition.

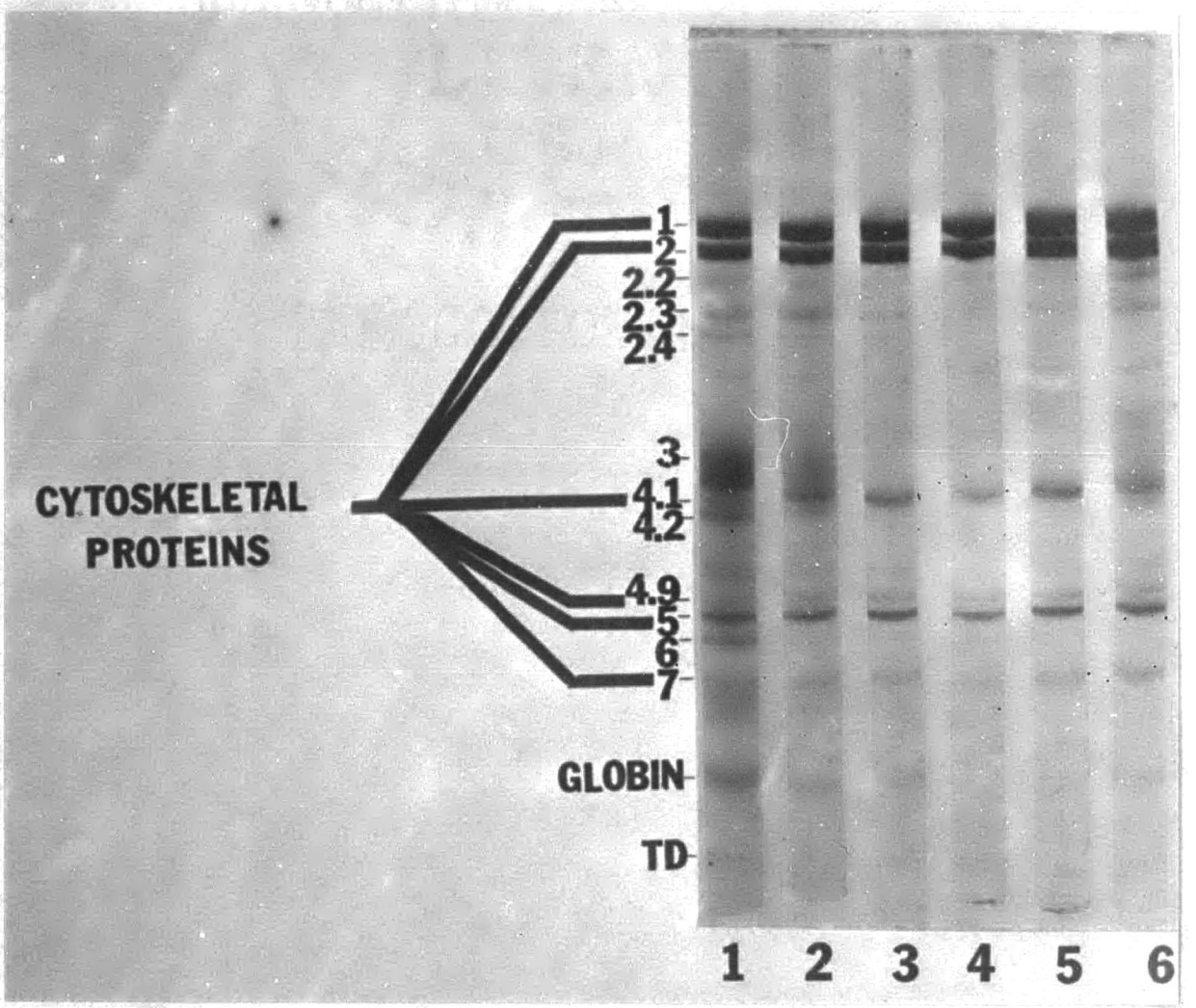
The Effect of Triton X-100, KCl and Proteolytic Inhibitors in Preparing Cytoskeletons

As the concentration of Triton X-100 was increased to 3%, non-cytoskeletal proteins were progressively removed, however, 5% Triton X-100 was no more effective than 3% Triton X-100 (Fig. 5). Because the volume of the 5% Triton X-100 insoluble fraction is significantly less than the 3% Triton X-100 insoluble fraction, and since we only obtain a small volume of ISC's after fractionation, the use of 3% Triton X-100 allowed us to obtain a visible pellet of ISC cytoskeletons.

Two concentrations of potassium chloride (KCl) were used to examine the effect of salt concentration: 6M KCl and 0.006 M KCl. It was noted by Sheetz (1979) that concentrations greater than 0.3M KCl were far more effective than low concentrations in removing non-cytoskeletal proteins. Analysis of cytoskeletal proteins with the above concentrations of KCl confirmed the findings of Sheetz et al. (Fig. 5, lanes 5 and 6).

It was suggested by Sheetz (1979) that pretreatment of cells and preparation of cytoskeletons in the presence of the proteolytic inhibitor Diisopropylphosphofluoridate (DFP) prevented the proteolytic degradation of non-cytoskeletal proteins (e.g., band 2.1), thereby enhancing their extraction with Triton X-100. Since there was evidence of a proteolytic degradation product of band 2.1, namely band 2.3, in cytoskeletons that were prepared in the absence of proteolytic inhibitors, the effect

Figure 5. Comparison of the effect of various concentrations of Triton X-100 and potassium chloride (KCl) on cytoskeleton protein composition. Cytoskeletons were subjected to electrophoresis as in Fig. 7. Gels: 1) Whole erythrocyte membranes; 2) Cytoskeletons prepared in 0.5% Triton X-100; 3) Cytoskeletons prepared in 1.5% Triton X-100; 4) Cytoskeletons prepared in 3% Triton X-100; 5) Cytoskeletons prepared in 5% Triton X-100 with 6M KCl; 6) Cytoskeletons prepared in 5% Triton X-100 with 0.006M KCl.


CYTOSKELETAL
PROTEINS
1
2
2.2
2.3
2.4
3
4.1
4.2
4.9
5
6
7
GLOBIN
TD
1
2
3
4
5
6

of DFP (20mM) in the pretreatment (Hepes-Ringer, pH 7.4) and the extraction buffer was examined. Neither band 2.1 nor 2.3 appeared in the protein profile of cytoskeletons prepared in the presence of DFP (Fig. 6). Despite DFP's effectiveness, it is not without great cost and some potential hazard (DFP is fatal if vapor is inhaled and through skin contact). Therefore, its usage was discontinued. Phenylmethylsulfonylfluoride (PMSF) and Trypsin inhibitors were also examined but proved to be ineffective in inhibiting proteolytic degradation (data not shown). Extraction was subsequently carried out in the absence of proteolytic inhibitors.

Comparison of Cytoskeletons Prepared From Isolated Membranes Versus Whole Cells

When cytoskeletons are prepared from membranes, it is conceivable that in the process of their isolation, several deleterious processes could occur, namely: (1) proteolytic degradation of membrane proteins due to protease activation (2) change in the ionic environment and subsequent change in protein-protein interactions. To avoid the occurrence of these processes, cytoskeletons were routinely prepared from intact cells rather than from membranes. To determine whether there was a difference in cytoskeletons from intact cells versus membranes, both were extracted employing the Sheetz's procedure. Analysis of both preparations on Fairbanks (4%) disc gels revealed that the two preparations were similar, though the cytoskeletons from intact cells contained an additional band, band 7, which was absent in membrane preparations (Fig. 7). This is consistent with what has previously been reported by Sheetz (1979).

SEPARATION OF IRREVERSIBLE SICKLED CELLS (ISC'S)

To analyze the protein composition of ISC cytoskeletons, it was necessary to isolate ISC's from non-ISC's. This was achieved when S/S whole cells were fractionated on a Dextran - 40 discontinuous gradient. Fractionation was based on density. ISC's have a higher density than normal cells, presumably due to the loss of

Figure 6. Effect of the presence of Diisopropylphosphofluoridate (DFP) in the pretreatment buffer (Hepes-Ringer, pH 7.4) and the extraction buffer on cytoskeletal protein composition. Cytoskeletons were subjected to electrophoresis as in Fig. 4. Gels: 1) Whole membranes; 2) Cytoskeletons prepared in the absence of DFP; 3) Cytoskeletons prepared in the presence of DFP.

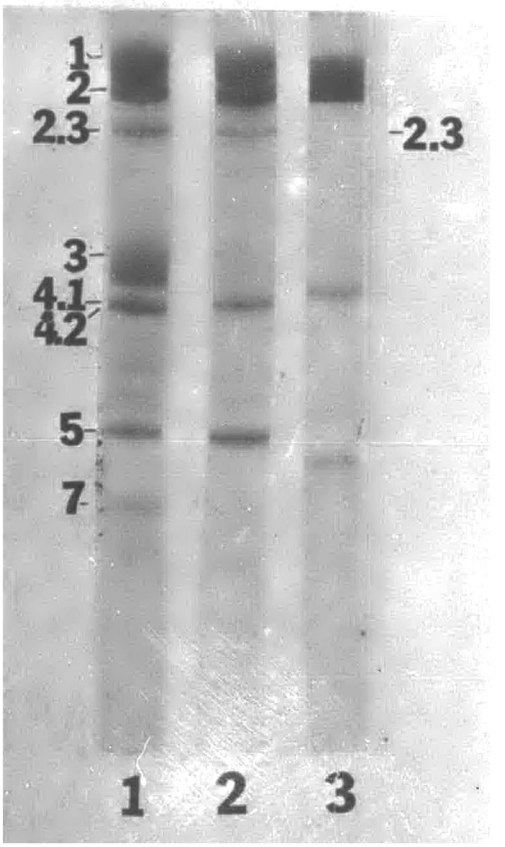
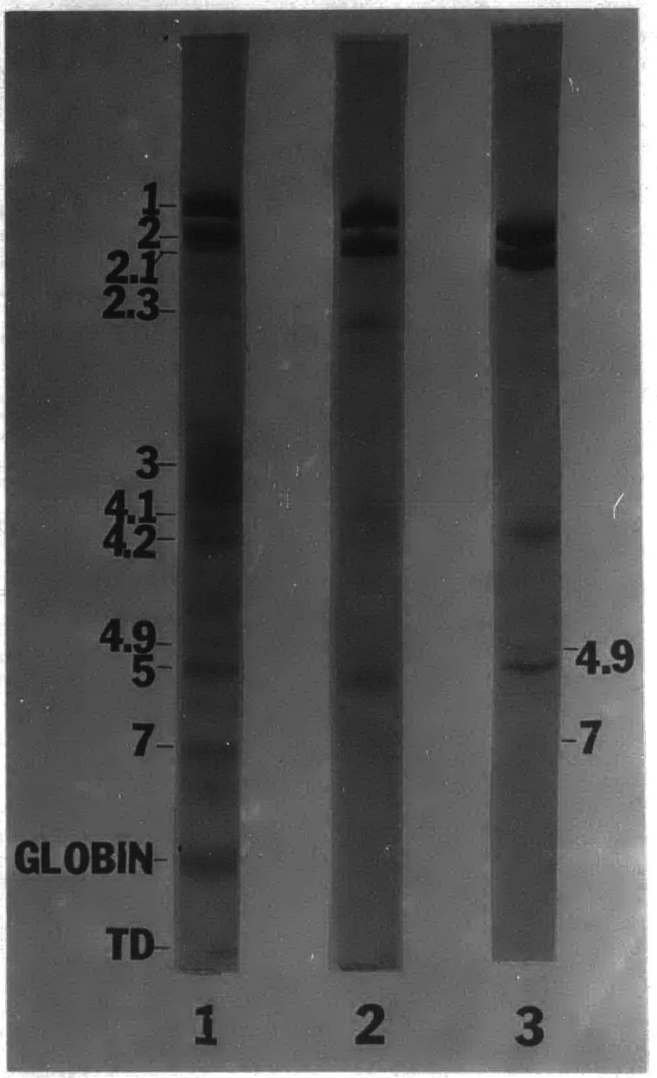

1
2
2.3
3
4.1
4.2
5
7
2.3
1
2
3

Figure 7. Comparison of cytoskeletal proteins prepared from membranes and intact cells. Cytoskeletons were subjected to electrophoresis as in Fig. 4. Gels: 1) Whole membranes; 2) Cytoskeletons extracted from membranes; 3) Cytoskeletons extracted from intact red blood cells.


1
2
2.1
2.3
3
4.1
4.2
4.9
5
7
GLOBIN
TD
4.9
7
1
2
3

water during the cascade process of ISC formation. A 60% Dextran cushion was utilized to avoid pelleting of cells. This allowed collection of ISC's from the side with an 18 gauge syringe. When the cushion was omitted, cells pelleted and adhered to the walls of the tube making collection difficult. Both normal and sickle blood yielded six bands of erythrocytes upon fractionation (Fig. 8). The bottom fraction of sickle blood always showed a greater intensity than the corresponding band in the normal blood indicating a greater number of dense cells. Each fraction was collected and treated as described in Methods.

Observation of erythrocytes in each fraction with the scanning electron microscope enabled visualization of cell shape and membrane surface texture (Figs. 9 and 10). As expected in the sickle blood, the percentage of ISC's increased dramatically from top to bottom fractions (top, 9% ISC's; middle, 20% ISC's; bottom, 71% ISC's). The sickled cells in each fraction were presumably ISC's since they retained the sickled shape after oxygenation for five min with 95% oxygen.

It has been reported that the surface of ISC's is often irregular but without visible organization (Lessin et al., 1978) when viewed with the scanning electron microscope and more recently, that some ISC's are echinocytic, i.e., have distinct surface irregularities (Palek et al., 1978). A small percentage of the cells in the bottom fraction appeared to be echinocytic but the majority of them had a nondescript shape with a slightly irregular surface. These observations are consistent with previous reports.

No difference was noted in the shape and texture of normal erythrocytes obtained from the top, middle and bottom fractions.

This analysis indicates that ISC's can readily be obtained by fractionating sickle cell erythrocytes in Dextran step-gradients.

Figure 8. Fractionation of normal and sickle erythrocytes in Dextran - 40 density gradients. (A/A), normal erythrocytes; (S/S), sickle erythrocytes. Top, middle and bottom fractions are denoted.

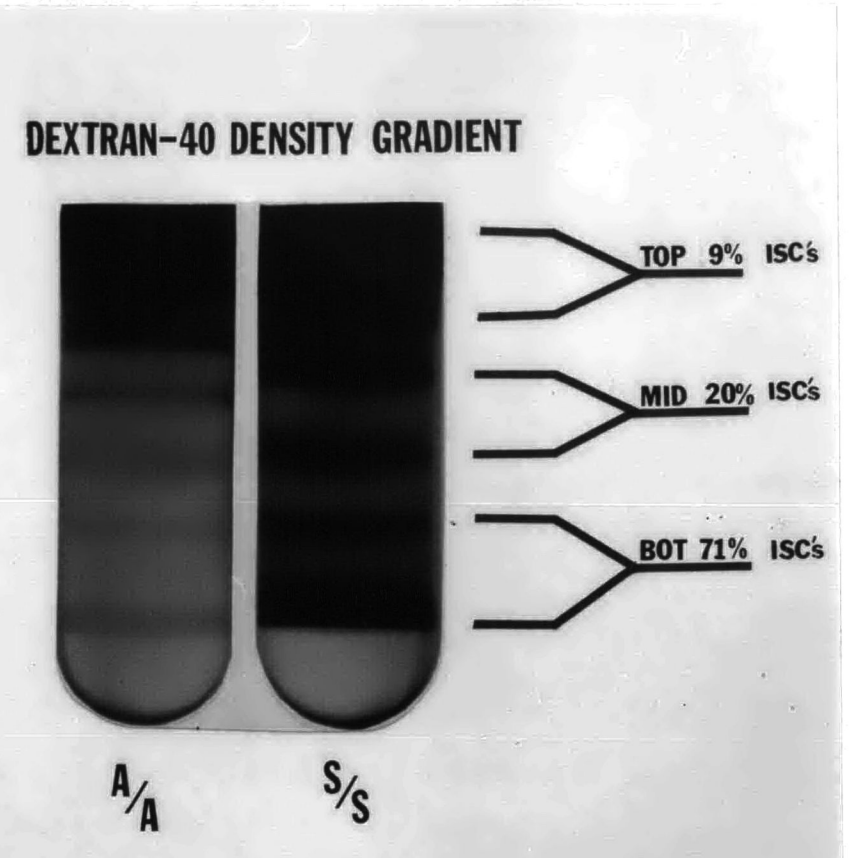

DEXTRAN-40 DENSITY GRADIENT
TOP 9% ISC's
MID 20% ISC's
BOT 71% ISC's
A/A
S/S

Figure 9. Scanning electron micrographs of fractions of normal and sickle erythrocytes from Dextran density gradients. Top (T), middle (M), bottom (B); normal (A/A), sickle (S/S). Magnification equals 4000X. Photographs were made with a model OS121 ETEC Omniscan Scanning Electron Microscope.

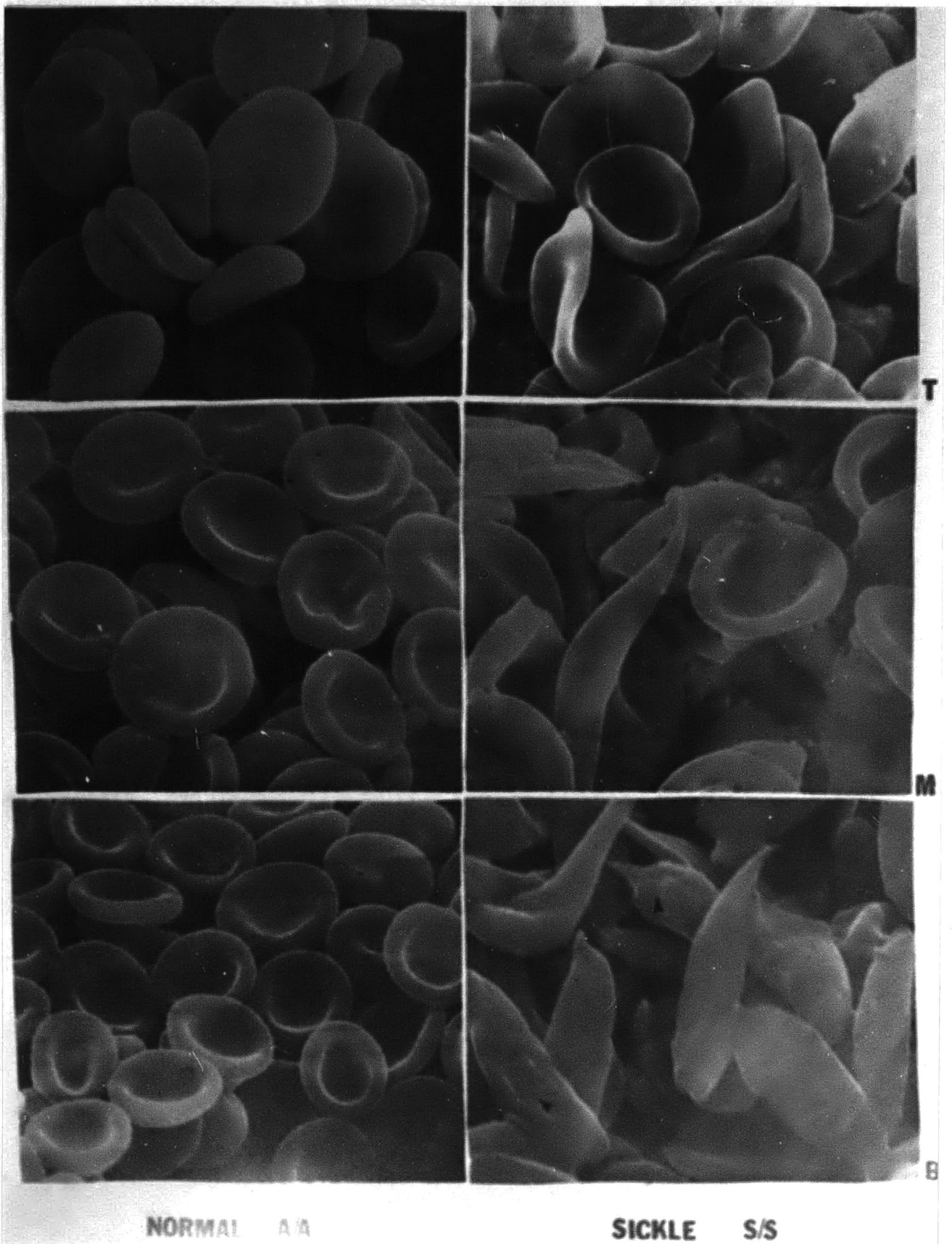
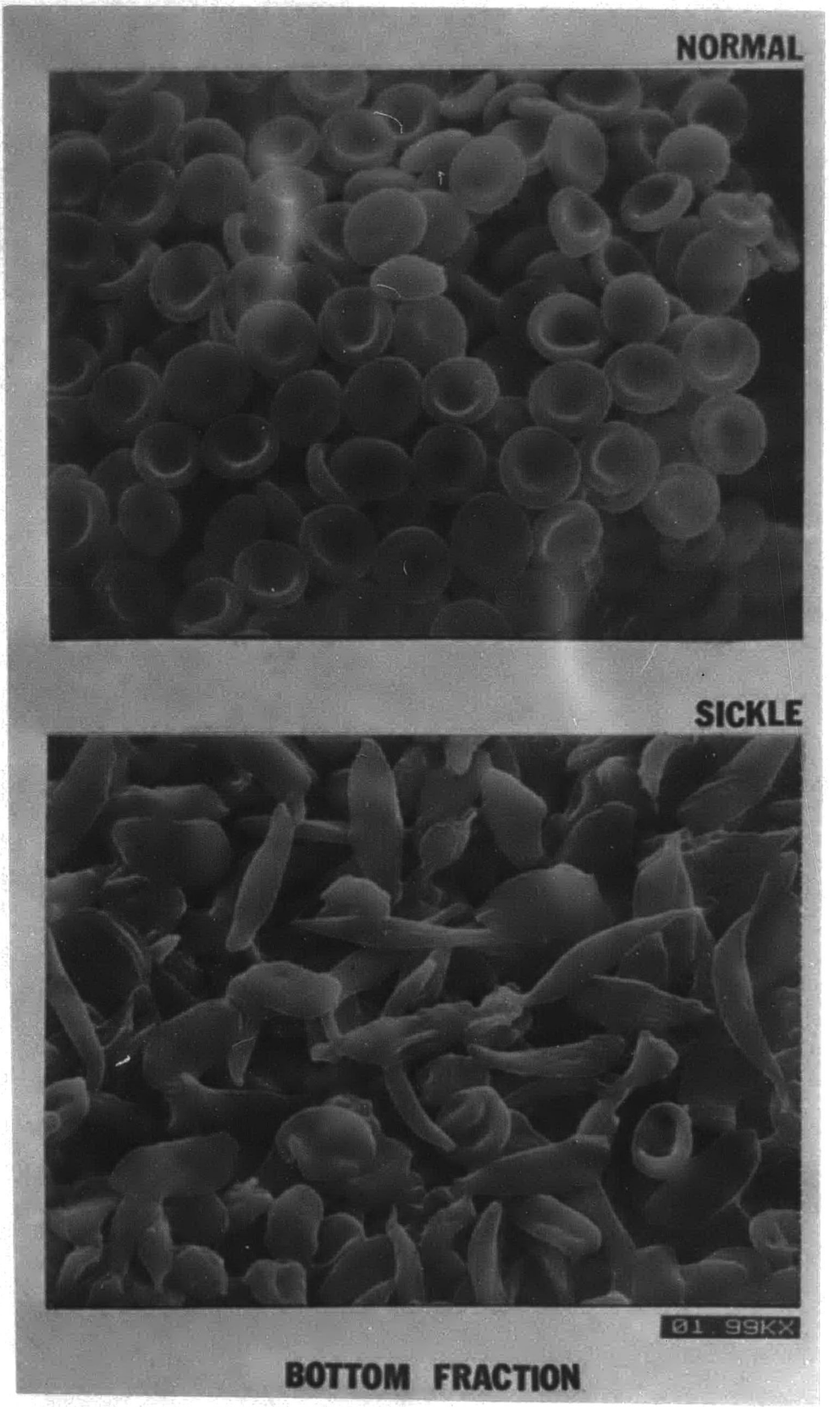

T
M
B
NORMAL A A
SICKLE S/S

Figure 10. Scanning electron micrographs of Dextran gradient bottom fractions of normal and sickle erythrocytes. Magnification, 2000X. Normal (A/A), sickle (S/S).

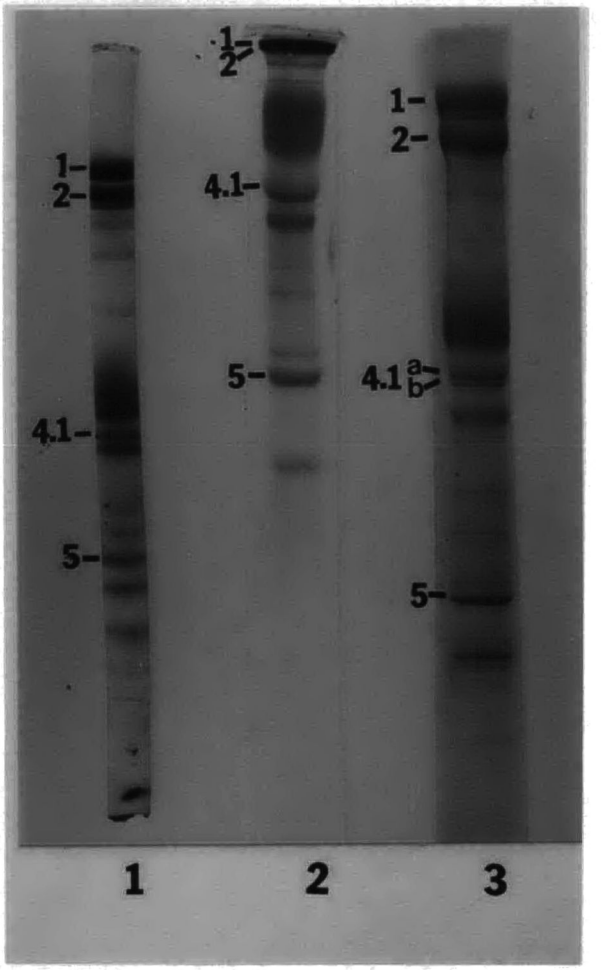

NORMAL
SICKLE
01 99KX
BOTTOM FRACTION

EXAMINATION OF ERYTHROCYTE MEMBRANE PROTEIN COMPOSITION AFTER SUBJECTING TO VARIOUS TYPES OF SDS-ELECTROPHORETIC AND STAINING PROCEDURES

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a technique that is widely used in research for fractionating membrane proteins. A previously reported electrophoretic analysis has not revealed a difference between ISC and normal cytoskeletal proteins (Lux et al., 1976). It is conceivable that the gel systems employed may not have been of adequate resolving power to detect differences between these two types of cytoskeletons. Recently, several refinements have been made in SDS-polyacrylamide gel electrophoresis and staining which permit greater resolution of proteins. In the following analysis, the resolving power of several procedures was analyzed. Total membrane proteins were subjected to electrophoresis using the following systems. (1) A Tris-Acetate continuous buffer system and 4% acrylamide disc gels (Fairbanks et al., 1971); (2) A Tris-Glycine Discontinuous Buffer System and 10% acrylamide slab gels (Laemmli, 1970); (3) A Tris-Glycine Discontinuous Buffer System and 5-15% acrylamide slab gradient gels (Laemmli, 1970).

One of the criteria used to determine the system of choice was the ability to separate band 4.1 (a cytoskeletal protein) into its individual components, bands 4.1a and 4.1b. These two polypeptides co-migrate on less resolving systems because of their close similarity in size. Band 4.1a has an estimated molecular weight of 80,000 daltons, whereas band 4.1b has an estimated molecular weight of 78,000 daltons. Fig. 11 shows membrane proteins fractionated using the three different electrophoretic systems described above. The Fairbanks system, the first system used successfully to fractionate membrane proteins, yields good resolution of the major membrane proteins however, bands 4.1a and 4.1b are not separated (Fig. 11, lane 1). The Laemmli 10% acrylamide system seemed to improve resolution in the region

Figure 11. Examination of normal erythrocyte membrane protein composition after various types of electrophoresis. Gels: 1) Fairbanks continuous 4% polyacrylamide disc gels; 2) Laemmli discontinuous 10% polyacrylamide slab gel; 3) Laemmli gradient 5-15% polyacrylamide slab gel.

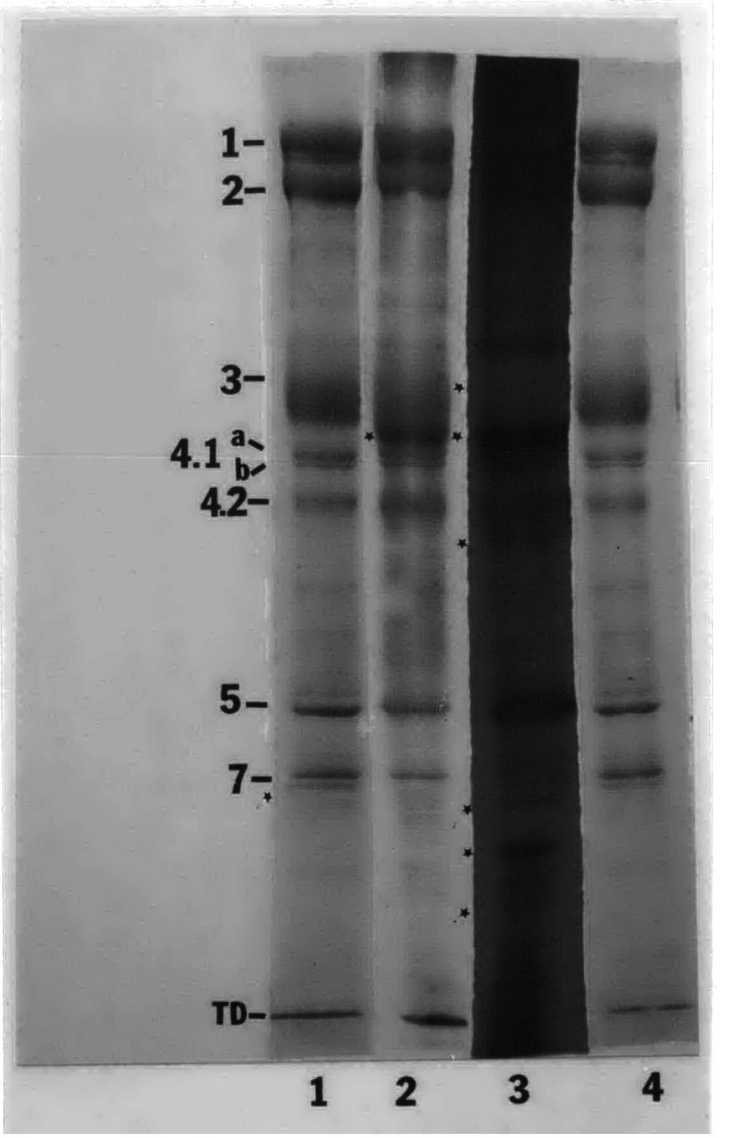

1
2
4.1
5
1
2
4.1
5
1
2
4.1a
b
5
1
2
3

between bands 4.1 and 5, but the high molecular weight proteins were not resolved very well (Fig. 11, lane 2). The Laemmli 5-15% acrylamide gel system gave the best resolution, resulting in the separation of 4.1a from 4.1b and at the same time yielding relatively good resolution in the high molecular weight regions of the gel (Fig. 11, lane 3). This latter system was subsequently routinely used to fractionate cytoskeletal and membrane proteins.

In an attempt to detect minor membrane proteins, two newly developed staining procedures were compared with the conventional staining procedure, which employs Coomassie blue. One of the stains examined, Silver, has been reported to be 100 times more sensitive than Coomassie blue (Merril et al., 1982); while the other stain, Gelcode was advertised (Upjohn Diagnostics) as having the potential to stain proteins different colors. This latter property could conceivably permit detection of differences between ISC and normal cytoskeletons.

Although some differences were observed, Silver stain and Gelcode did not reveal many additional bands (Fig. 12). When gels were stained with Silver, in contrast to Coomassie blue, diffuse staining was observed at the top of the gel and a prominently stained band was observed between bands 3 and 4.1 (compare lane 2 with 1 and 4). These regions are unstained in gels stained with Coomassie blue. Since all of the bands detected with Coomassie blue are also detected with Silver stain, the detection of additional staining regions increases our ability to detect differences between sickle and normal membranes. The two regions stained with Silver but not Coomassie blue are probably glycoproteins, since Silver stains glycoproteins more readily than Coomassie blue (Merril et al., 1982). The band between bands 3 and 4.1 seems to be in the position of Glycophorin A (PAS-1), adding support to the hypothesis that the additional bands detected with Silver are glycoproteins. Interestingly, band 3 does not stain very intensely when this stain is used.

Figure 12. Examination of various protein staining procedures. Erythrocyte membrane proteins electrophoresed on Laemmli gradient (5-15%) gels stained with Gels: 1) Coomassie blue; 2) Silver; 3) Gelcode; 4) Coomassie blue. Asterisks indicate differences.


1–
2–
3–
4.1 a
b
4.2–
5–
7–
TD–
1
2
3
4

Gels stained with Gelcode have a very interesting appearance but do not photograph well (Fig. 12, lane 3). The major erythrocyte proteins, bands 1, 2 and 3 do not stain very well, although the other proteins detected by Coomassie blue are stained (compare lanes 3 and 4). The band between bands 3 and 4.1 detected with Silver, is also stained with Gelcode and several additional bands below band 7 are seen in the Gelcode-stained gel but not the Coomassie blue stained gels. These latter bands seem to be present in the Silver-stained gel although they are not stained as intensely. All three stains were subsequently used to search for alterations in the protein composition of ISC cytoskeletons.

COMPARATIVE ANALYSIS OF CYTOSKELETAL PROTEINS OF NORMAL (A/A), IRREVERSIBLE AND NON-IRREVERSIBLE SICKLED CELLS, UTILIZING SDS-GEL ELECTROPHORESIS

To detect possible differences in the protein composition of ISC and normal erythrocyte cytoskeletons, cytoskeletal complexes were prepared from the top, middle and bottom dextran gradient fractions of sickle and normal erythrocytes and subjected to electrophoresis on SDS-polyacrylamide Laemmli gradient (5-15%) gels. Gels were subsequently stained with Coomassie blue, Silver and Gelcode. Cytoskeletons from cells in each of the different fractions were composed of the cytoskeletal components: Spectrin, (bands 1 and 2), band 4.1 (a and b) and band 5 (actin). In addition, other proteins were noted including the ankyrin family, band 7 and band 4.2. These latter proteins are frequently observed when cytoskeletons are prepared from whole cells (Fig. 13). Each cytoskeletal fraction had the same protein composition. Thus, no differences in either major or minor components were observed after staining with Coomassie blue.

When another aliquot of this same sample was electrophoresed under identical conditions and the gels stained with Silver (Fig. 14), no differences were detected

Figure 13. Comparison of cytoskeletal proteins from top, middle and bottom fraction erythrocytes (normal and sickle) on Laemmli gradient (5-15%) polyacrylamide slab gels, after staining with Coomassie blue. Lanes: 1) Whole membranes (A/A); 2) Whole membranes (S/S); 3) Top fractions, normal cytoskeleton; 4) Middle fraction, normal cytoskeleton; 5) Bottom fraction, normal cytoskeleton; 6) Top fraction, sickle cytoskeleton; 7) Middle fraction, sickle cytoskeleton; 8) Bottom fraction, sickle cytoskeleton.

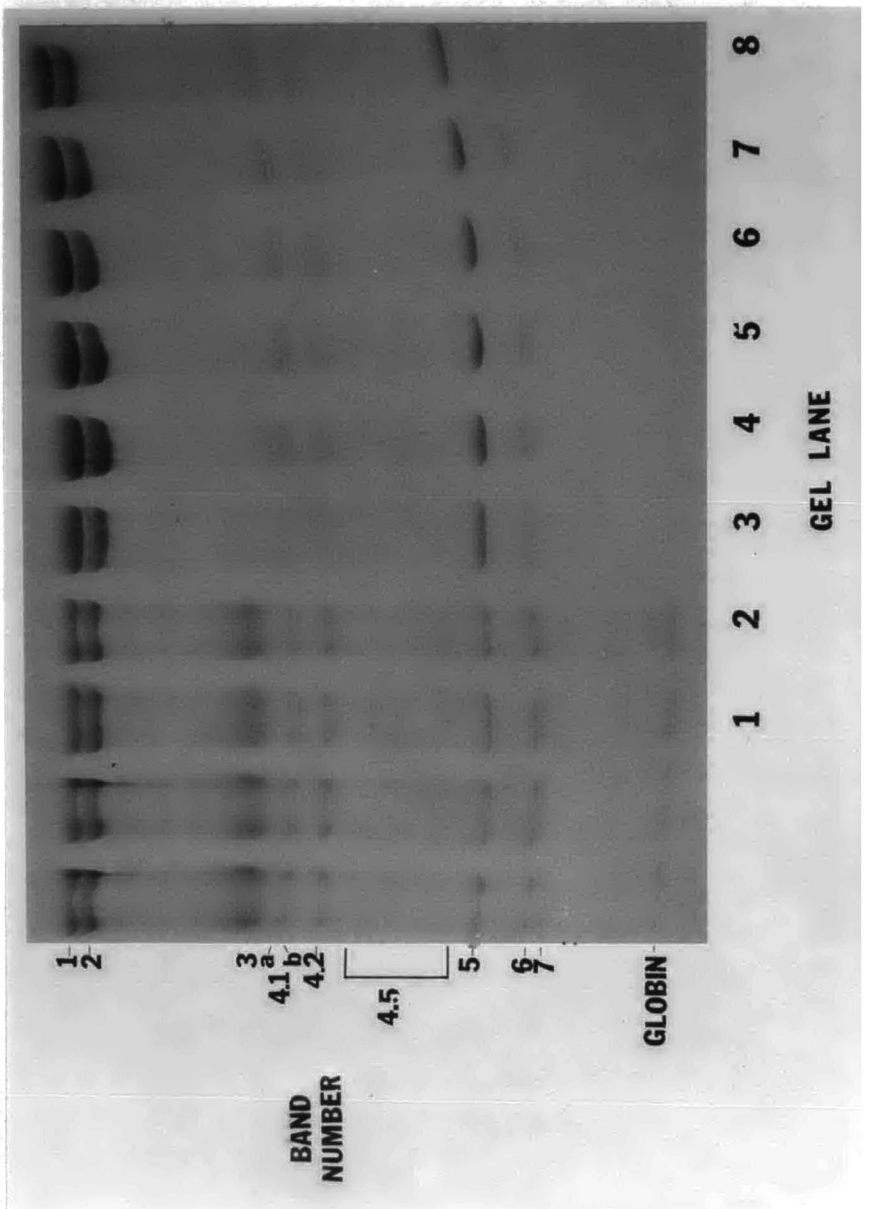
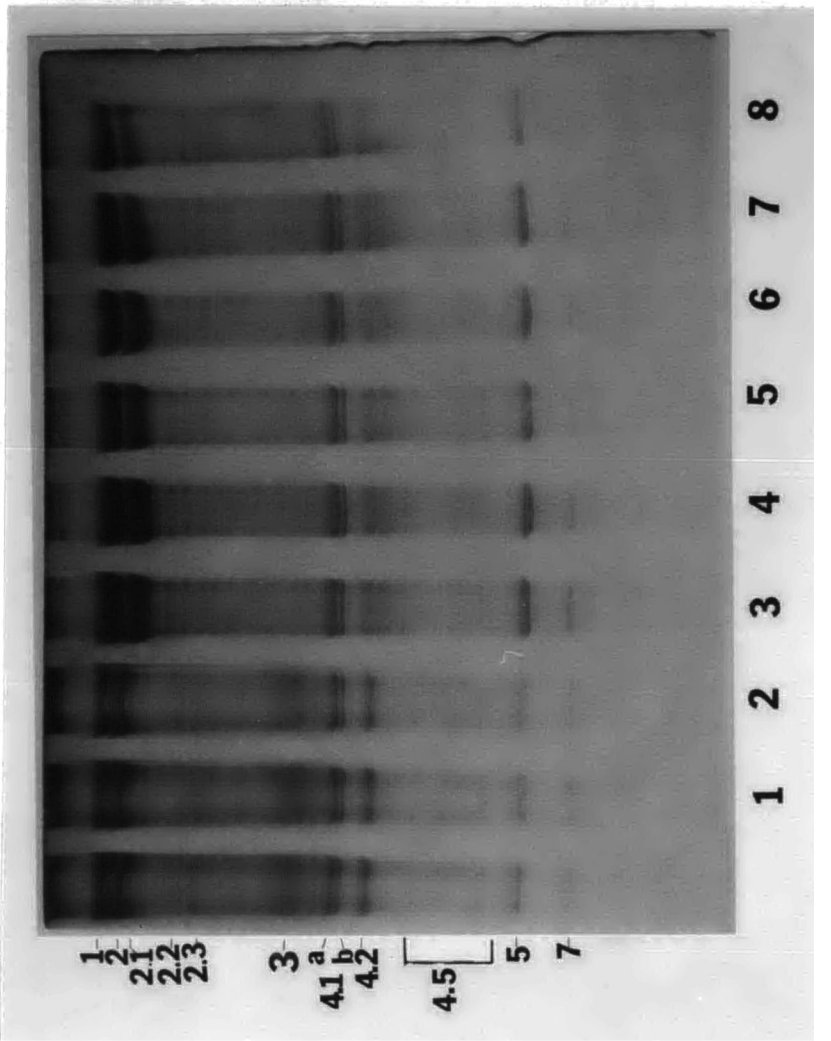

1
2
3
4.1a
4.1b
4.2
4.5
5
6
7
GLOBIN
BAND NUMBER
1 2 3 4 5 6 7 8
GEL LANE

Figure 14. Comparison of cytoskeletal proteins from top, middle and bottom fraction erythrocytes (normal and sickle) on Laemmli gradient polyacrylamide slab gels, after staining with Silver. Electrophoresis was carried out as described in Fig. 13.


1
2
2.1
2.2
2.3
3
4.1 a
b
4.2
4.5
5
7
1 2 3 4 5 6 7 8

between the different fractions, though minor proteins were more visible in the 2.1 region. The bands detected in whole membranes with Silver but not Coomassie blue do not appear to be associated with the cytoskeleton, again suggesting that they are glycoproteins. Although band 7 seems to be absent in lanes 5-8, this is probably an artifact of photography since it was detected in all lanes after Coomassie blue staining (Fig. 13).

As in the above cases, when cytoskeletal proteins from the different fractions were stained with Gelcode after electrophoresis, no differences were observed (Fig. 15), however of great interest, bands were observed which were not seen after staining with either Coomassie blue or Silver. The lower part of the region between 4.2 and 5 contained several negatively stained bands and the region below band 7, several positively stained bands. These observations suggest that the cytoskeleton may be more complex than presently imagined (Lux, 1979).

COMPARATIVE ANALYSIS OF CYTOSKELETAL PROTEINS OF NORMAL AND IRREVERSIBLE SICKLED CELLS UTILIZING ISOELECTRIC FOCUSING AND TWO DIMENSIONAL ELECTROPHORESIS

Isoelectric Focusing (IEF)

SDS-Polyacrylamide Gel Electrophoresis, the technique used in the preceeding studies, fractionates proteins on the basis of size. Isoelectric focusing is another analytical tool that is extremely useful for fractionating proteins species from complex mixtures based on net charge.

The cytoskeletal preparations (sickle and normal) were focused over a pH range of 2.9 (acidic end) to 7.1 at the (basic end) (Fig. 16-A). The pH gradient was determined by a single contact electrode read with the standard pH meter.

Several modifications were made in the solubilizing buffer and electrophoresis conditions during the course of this study (data not shown). The resolution

Figure 15. Comparison of cytoskeletal proteins from top, middle and bottom fraction erythrocytes (normal and sickle) on Laemmli gradient polyacrylamide slab gels, after staining with Gelcode. Electrophoresis was carried out as described in Fig. 13.

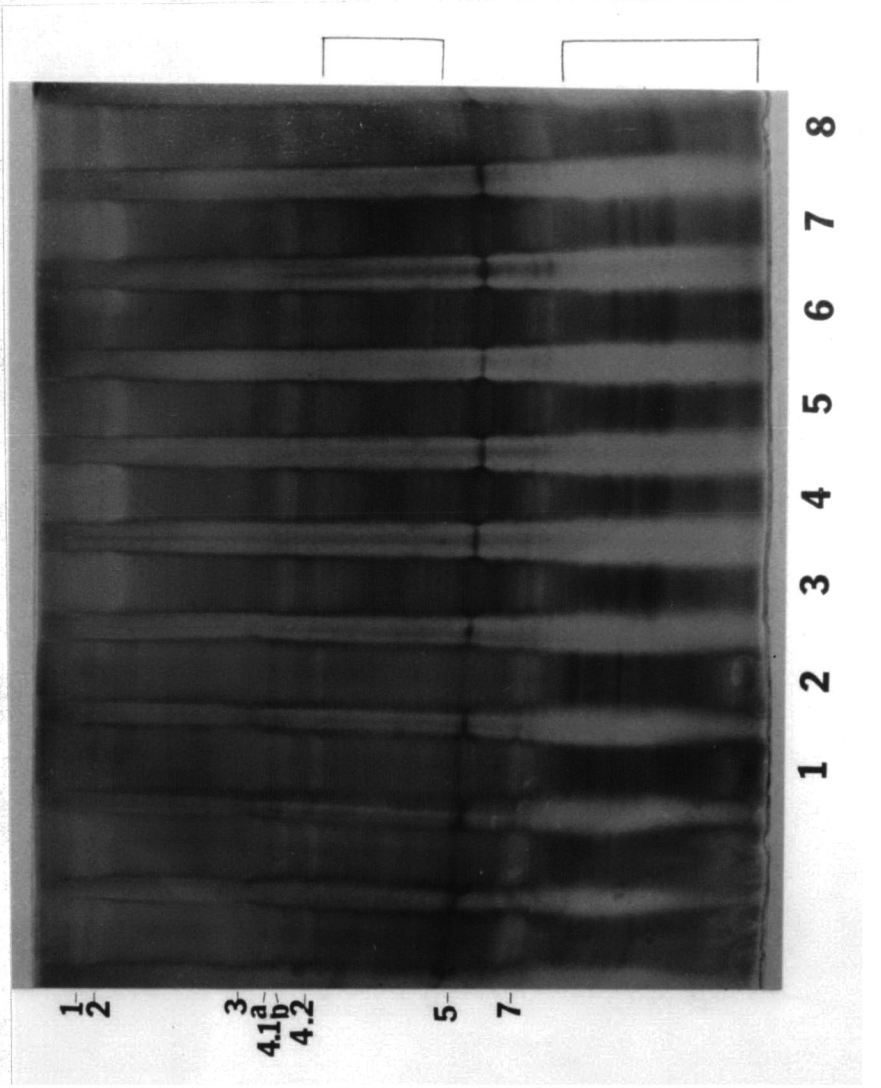
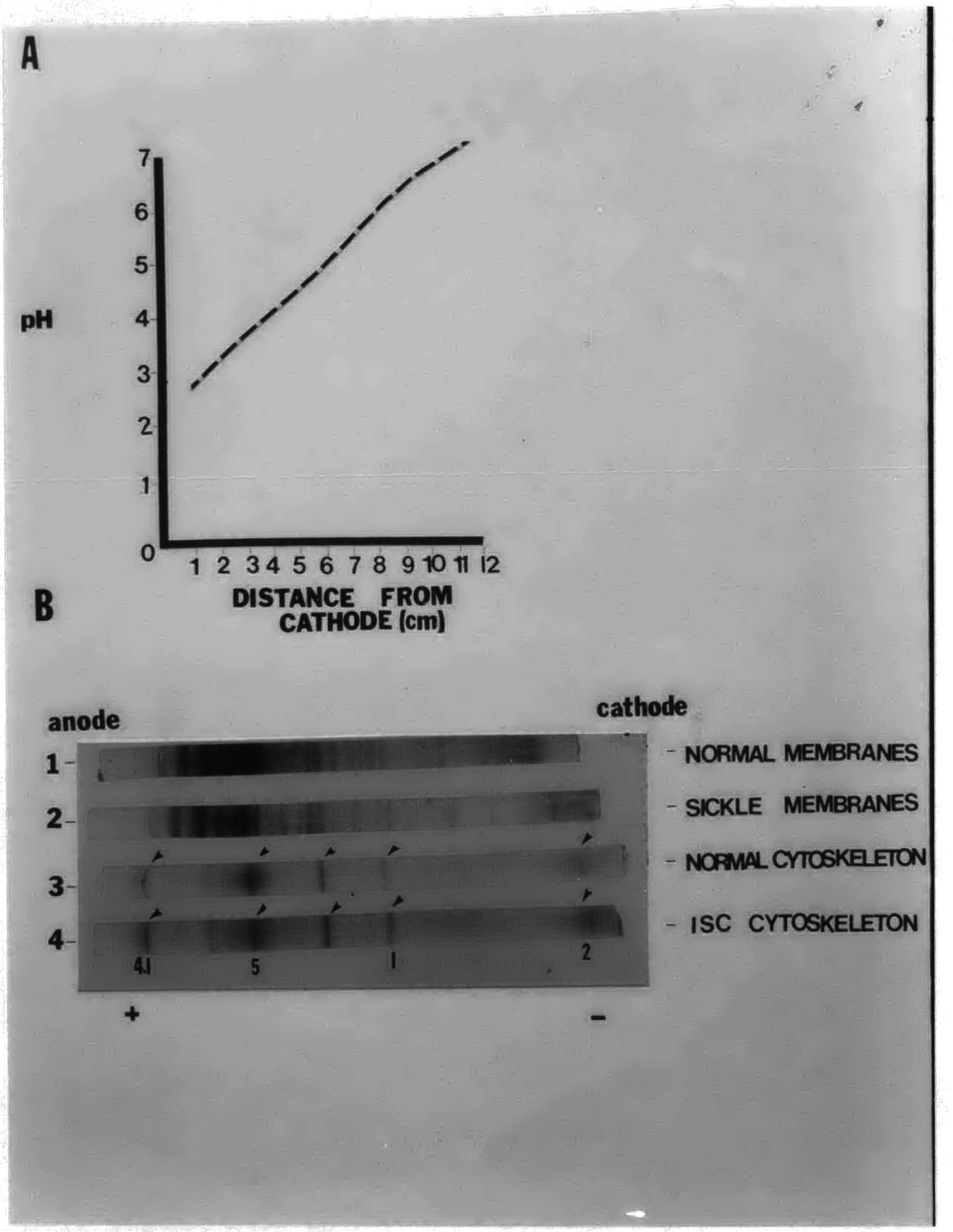

1
2
3
4.1a
b
4.2
5
7
1 2 3 4 5 6 7 8

Figure 16. Isoelectric focusing of ISC and normal cytoskeletons and whole membrane proteins. (A) pH gradient of blank gel following electrophoresis. (B) IEF of cytoskeleton and membrane proteins: 1) Normal cell membrane proteins; 2) ISC membrane proteins; 3) Normal cell cytoskeletal proteins; 4) ISC cytoskeletal proteins.


A
pH
7
6
5
4
3
2
1
0
1 2 3 4 5 6 7 8 9 10 11 12
DISTANCE FROM CATHODE (cm)
B
anode
cathode
1
2
3
4
4.1
5
1
2
+
-
NORMAL MEMBRANES
SICKLE MEMBRANES
NORMAL CYTOSKELETON
ISC CYTOSKELETON

ultimately achieved was considered good (Fig. 16-B).

When ISC and normal cytoskeletal preparations were compared on IEF gels, their protein profiles appeared identical (Fig. 16-B, lanes 4 and 5). Approximately ten bands were visualized in each preparation. The major cytoskeletal polypeptides i.e., bands 1, 2, 4.1 and 5 have been tentatively identified in the gel based on their mobility when the gels were subjected to SDS gel electrophoresis, perpendicularly to their long axis (two-dimensional electrophoresis).

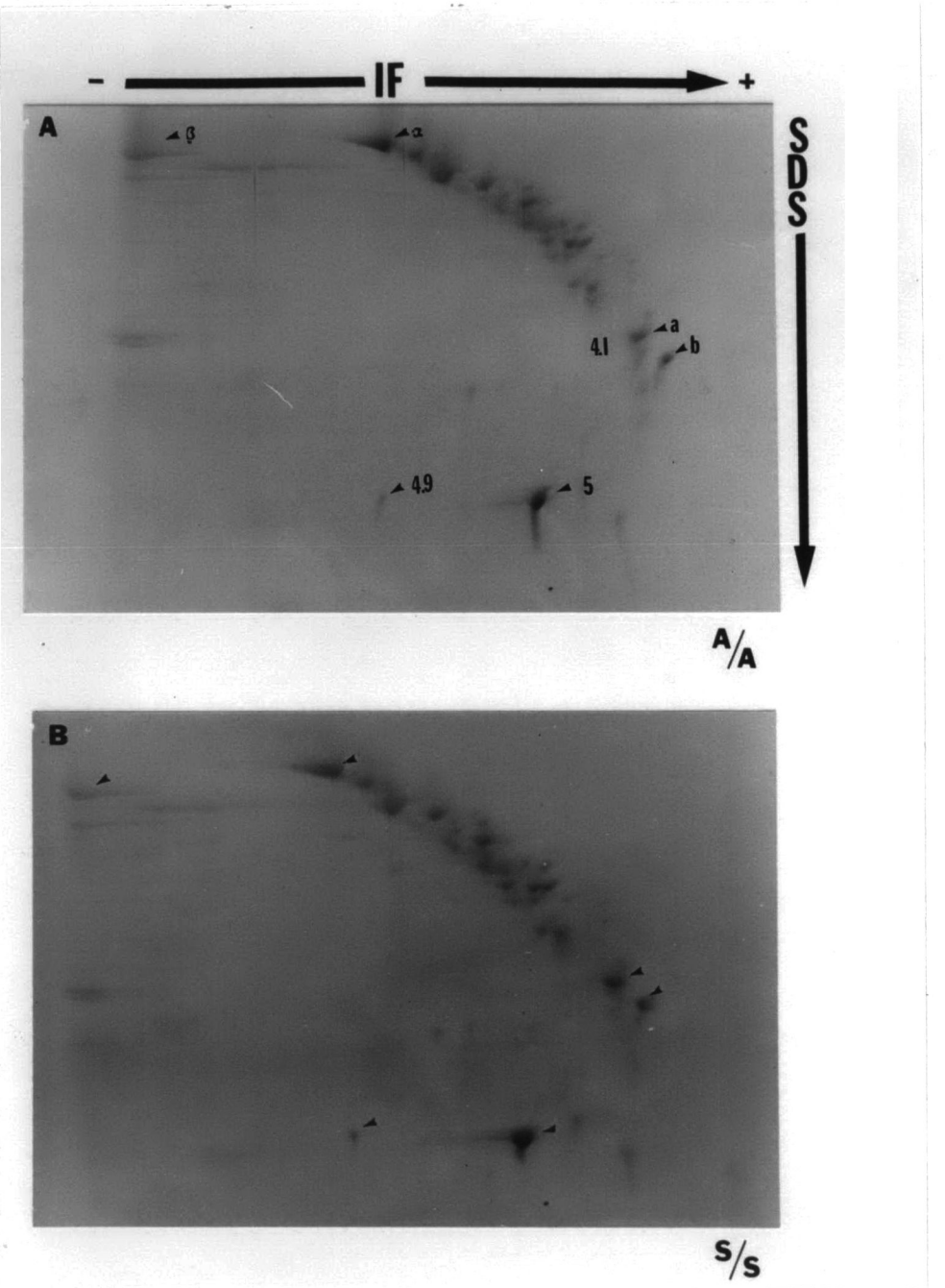
The relatively simple protein profile of the cytoskeletal preparations versus the more complex profile of the whole membrane preparations suggests that charge modification, a frequently encountered problem associated with IEF, is not occurring. It is of considerable interest, in view of the many similarities between bands 1 and 2, that they seem to possess quite different isoelectric points (pIs).

Two-Dimensional Electrophoresis (IEF/SDS PAGE)

This technique employs both Isoelectric Focusing (IEF) and SDS-Polyacrylamide gels (SDS-PAGE) to fractionate complex mixtures of proteins. The fact that proteins are separated according to two parameters improves resolution when compared to either one of the one-dimensional techniques.

Two-dimensional analysis of normal or ISC preparations revealed approximately 40 different spots (Fig. 17), some of which were easily detected, while others were somewhat obscure. Some of the spots have been identified, based on their mobility in the second-dimension (SDS), e.g., actin is found in the lower plane of the two-dimensional gel. The large number of spots seen in the two-dimensional electrophoretogram is probably artifactual, since the cytoskeleton appears to contain only five polypeptides when examined by SDS-gel electrophoresis (bands 1, 2, 4.1, 5 and 7), three of which (bands 1, 2 and 5) appear to be single polypeptides and thus, should not be further fractionated by subjecting the sample to IEF.

Figure 17. Two-dimensional electrophoresis (IEF/SDS-PAGE) of cytoskeletal proteins (bottom fraction, normal and sickle). (A) Normal cytoskeletons; (B) ISC cytoskeletons. Large arrows indicate the direction in which the proteins moved. Small arrows denote the major cytoskeletal proteins, i.e., bands 1, 2, 4.1a,b and 5.


-
IF
+
A
β
α
SDS
a
b
4.1
4.9
5
A/A
B
S/S

No significant differences between the ISC and normal cytoskeleton preparation were observed.

COMPARATIVE ANALYSIS OF THE ORGANIZATION OF PROTEINS IN THE ISC AND NORMAL CYTOSKELETON USING CROSSLINKING REAGENTS

It is probable that the proteins in ISC cytoskeletons are arranged differently from those in normal biconcave erythrocyte cytoskeletons. To search for organizational differences, a battery of crosslinking reagents was employed. Crosslinking reagents are bifunctional molecules or catalysts which are able to covalently link two closely associated proteins by serving as a bridge between or catalyzing the covalent linkage of two similar chemical groups in the two proteins. A number of these reagents are available commercially varying in reactive groups and size. Fig. 18 shows the chemical structure of the crosslinking molecules employed in this study. The asterisks point out the reactive groups for that particular molecule.

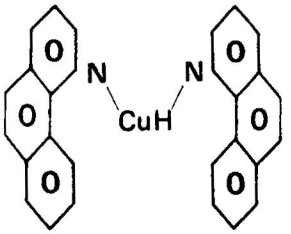
Preliminary experiments were conducted to determine the effective concentration and reactive conditions for each crosslinking reagent (data not shown). In the studies reported here, ISC-rich and normal cytoskeletons were incubated with one concentration of each crosslinking reagent and the crosslinked products examined as a function of time.

O-phenanthroline/Copper Sulfate

O-phenanthroline catalyzes the oxidation of sulfhydryl groups leading to the formation of disulfide bonds between two such groups. Crosslinked complexes are located at the top of the gel (depicted by arrows) and were observed even at 0 time in both sickle and normal preparations (Fig. 19). There was a concommitant reduction in bands 1, 2, 2.1, 4.1 and 7 as the time of incubation was increased, indicating that these polypeptides were being crosslinked. Band 5 did not appear to

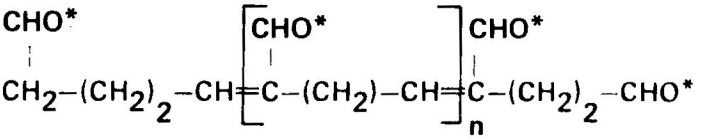
Figure 18. Chemical structures of the crosslinking reagents used in this study (A) O-phenanthroline/Copper Sulfate (O-phe); (B) Glutaraldehyde (Glute); (C) Dithiobis (succinimidyl propionate) (DTSP); (D) Dimethyl 3,3'dithiobispropionimidate-2HCl (DTBP); (E) Dimethyladipimidate (DMA); and (F) Dimethylsuberimidate (DMS). Asterisks indicate the reactive groups for each molecule. Arrows indicate bonds which can be cleaved by reducing agents (e.g., Dithiothreitol (DTT) or 2-β-mercaptoethanol) to identify crosslinked products.

(A)



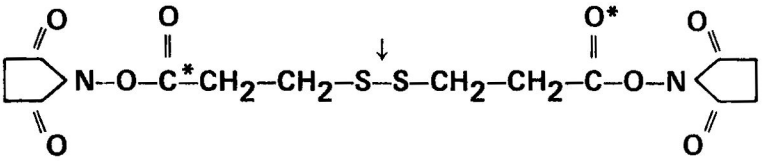
O–phenanthroline/Copper Sulfate (O–phe)

(B)



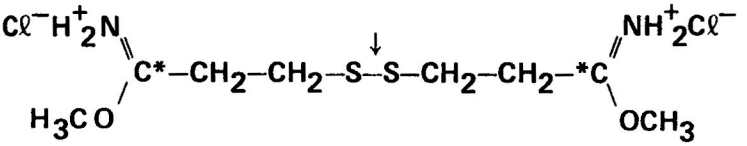
Glutaraldehyde (Glute)

(C)



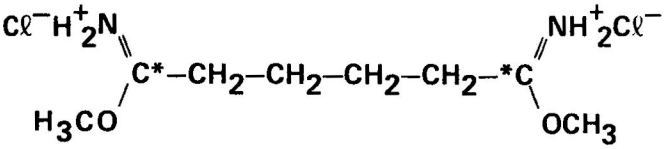
Dithiobis(Succinimidylpropionate) (DTSP)

(D)



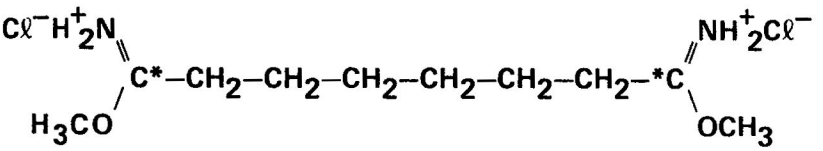
Dimethyl 3,3'–dithiobispropionimidate–2HCℓ (DTBP)

(E)



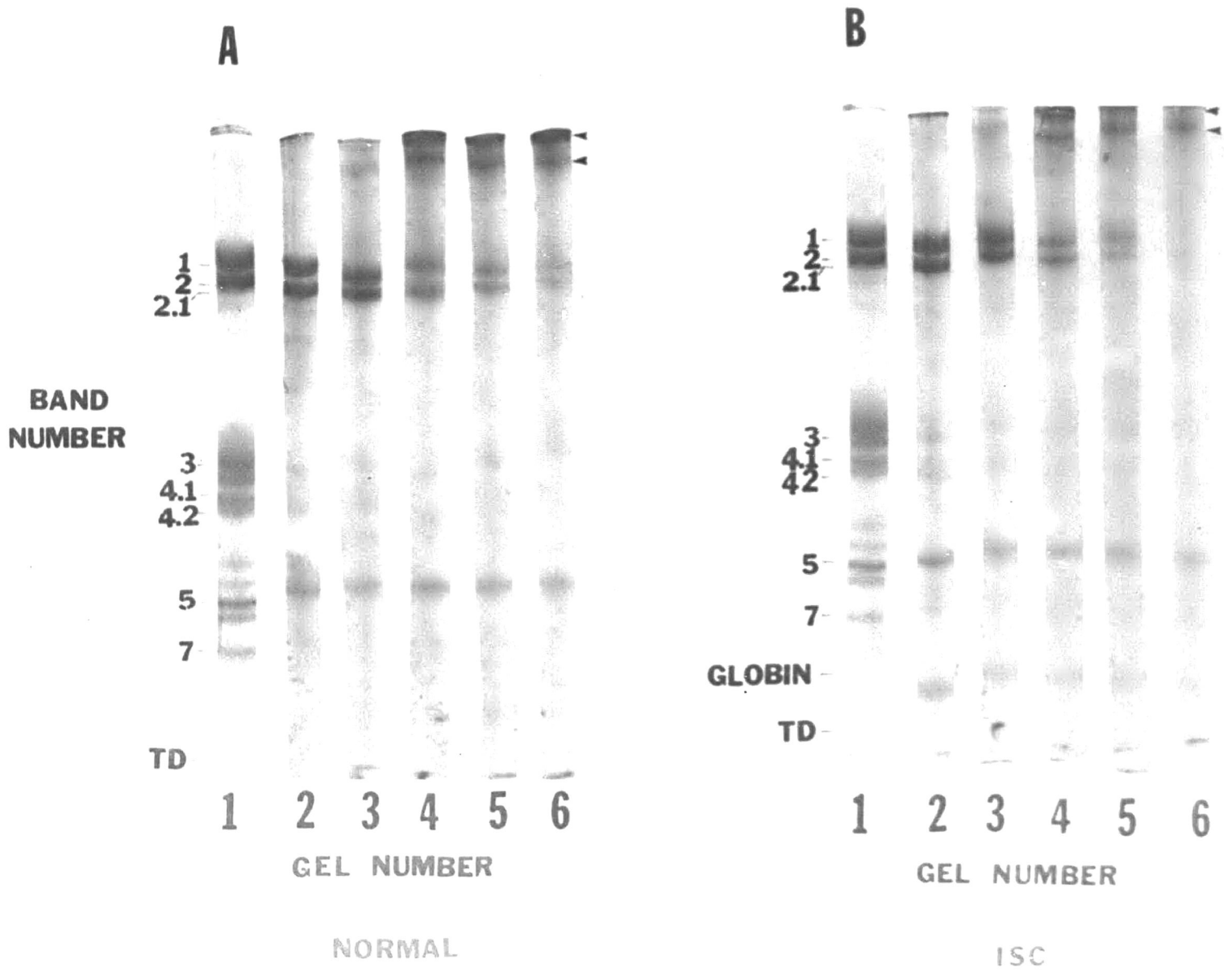
Dimethyladipimidate (DMA)

(F)



Dimethylsuberimidate (DMS)

Figure 19. Crosslinking of cytoskeletons from (A) Normal and (B) ISC-rich cells with 5mM O-phe/$CuSO_4$. Electrophoresis was carried out on 4% Fairbanks acrylamide gels, and the gels stained with Coomassie blue. Gels: 1) Control membranes; 2) Control cytoskeletons; 3) Cytoskeletons crosslinked for 0 min; 4) Cytoskeletons crosslinked for 10 min; 5) Cytoskeletons crosslinked for 20 min; 6) Cytoskeletons crosslinked for 30 min. Arrows at the top of the photographs indicate crosslinked products.


A
BAND NUMBER
1
2
2.1
3
4.1
4.2
5
7
TD
1 2 3 4 5 6
GEL NUMBER
NORMAL
B
1
2
2.1
3
4.1
4.2
5
7
GLOBIN
TD
1 2 3 4 5 6
GEL NUMBER
ISC

be crosslinked in either preparation.

There seemed to be no difference between ISC and normal preparations in either the kinetics of crosslinking or the crosslinked products which were formed. Thus, this crosslinking reagent does not reveal a difference in the organization of proteins in ISC and normal cytoskeletons.

Interestingly, hemoglobin was associated with the ISC-rich cytoskeleton and appeared to be crosslinked as time increased.

Glutaraldehyde

Glutaraldehyde forms amide bridges between amino and carbonyl groups of neighboring proteins. The crosslinked products were so large that they just entered the gel (Fig. 20). Bands 1, 2, 2.1 and 4.1 appeared to be rapidly crosslinked, while Band 7 seemed to be crosslinked relatively slowly. It is of interest that band 1 seemed to be crosslinked faster than 2, since 1 and 2 are intimately associated in the cytoskeleton as dimers. As observed in the case of O-phenanthroline, band 5 was not crosslinked to any significant extent.

Here again, no differences were detected between ISC and normal cytoskeletal preparations, in either the kinetics of crosslinking or the crosslinked products formed.

Dimethyl 3,3'- Dithiobispropionimidate (DTBP)

DTBP belongs to a class of crosslinking reagents called imidoesters. They specifically react with proteins by amidination of primary amino groups.

Most of the crosslinked products produced with this reagent failed to enter the gel, however a small fraction was contained within the gel (Fig. 21). Bands 1 and 2 showed a gradual reduction as time was increased but not as dramatically as when exposed to other crosslinking reagents. Though 4.1 appeared in relatively low concentration in the preparations (untreated cytoskeleton control), it is clear that

Figure 20. Crosslinking of cytoskeletons from (A) Normal and (B) ISC's with 5mM Glutaraldehyde. Electrophoresis was carried out on 4% Fairbanks gels, and the gels stained with Coomassie blue. Gels: 1) Control membranes; 2) Control cytoskeletons; 3) Cytoskeletons crosslinked for 0 min; 4) Cytoskeletons crosslinked for 1 min; 5) Cytoskeletons crosslinked for 2 min; 6) Cytoskeletons crosslinked for 3 min. Arrows at the top of the photographs indicate crosslinked products.

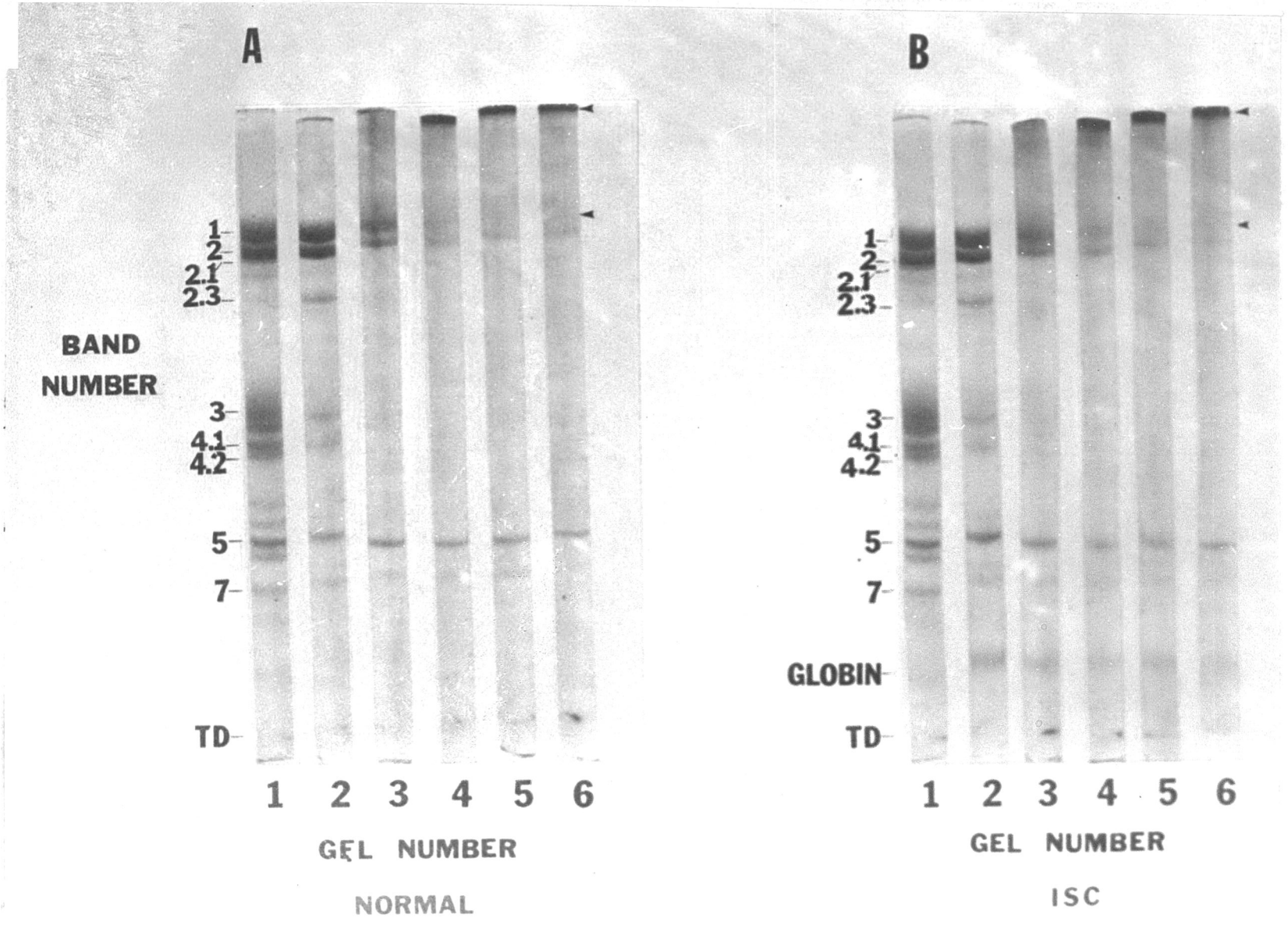
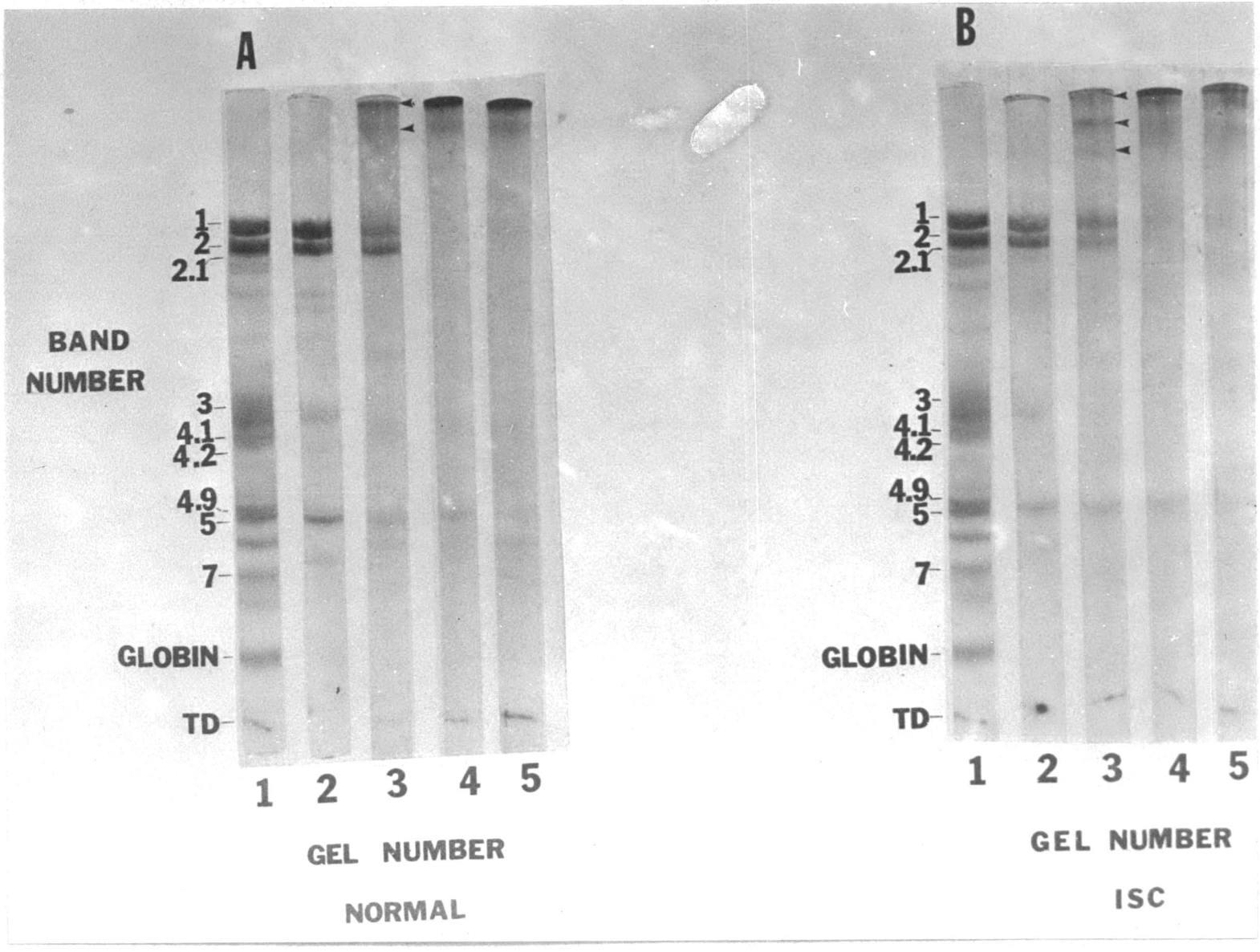

A
B
BAND NUMBER
1
2
2.1
2.3
3
4.1
4.2
5
7
GLOBIN
TD
1 2 3 4 5 6
GEL NUMBER
NORMAL
ISC

Figure 21. Crosslinking of cytoskeletons from (A) Normal and (B) ISC's with 1mM Dithiobis (succinimidyl propionate) (DTSP). Electrophoresis was carried out on 4% Fairbanks gels, and the gels stained with Coomassie blue. Gels: 1) Control membranes; 2) Control cytoskeletons; 3) Cytoskeletons crosslinked for 0 min; 4) Cytoskeletons crosslinked for 1 min; 5) Cytoskeletons crosslinked for 2 min. Arrows at the top of the photographs indicate crosslinked products.


A
B
BAND NUMBER
1
2
2.1
3
4.1
4.2
4.9
5
7
GLOBIN
TD
1 2 3 4 5
GEL NUMBER
NORMAL
ISC

there was a rapid reduction or total disappearance of this band as a function of time (Fig. 21, lane 6). Band 5 did not appear to be reduced, and therefore remained uncrosslinked even under the most extreme crosslinking conditions.

No differences were observed between the ISC and normal cytoskeletal preparations.

Dithiobis (Succinimidyl Propionate) (DTSP)

DTSP is a cleavable imidoester, which reacts similarly to DTBP. DTSP crosslinked products constituted two to three bands in the upper quadrant of the gel and also protein complexes which were too large to enter the gel (Fig. 22). All of the cytoskeletal polypeptides appeared to be components of the crosslinked products including band 5 as evidenced by the reduction in the intensity of bands.

There appeared to be a difference in the crosslinked products produced in the sickle and normal cytoskeletons. At 0 time, two crosslinked products seemed to be common to both preparations, but there was a third unique band in the ISC cytoskeleton. Other differences were noted after two min of reaction suggesting that the crosslinking reaction was proceeding at a slower rate in the ISC preparation; while these differences are interesting, it was not observed when two other ISC cytoskeletal preparations were compared with normal after reacting with DTSP. Thus, the significance of this observation is unclear.

Dimethyladipimidate (DMA)

DMA is an imidoester which like DTBP, reacts with primary amino groups, forming amide linkages. The distance between reactive groups is approximately 8.6 angstroms (Å) whereas in DTBP it is approximately 11 Å.

DMA was relatively ineffective in crosslinking cytoskeletal proteins (Fig. 23). Although some polypeptides must have been crosslinked, based on the fact that crosslinked products were present, a reduction in cytoskeletal polypeptides was not

Figure 22. Crosslinking of cytoskeletons from (A) Normal and (B) ISC's with 10mg/ml Dimethyl 3,3'-dithiobispropionimidate - 2HCl (DTBP). Electrophoresis was carried out on 4% Fairbanks gels, and the gels stained with Coomassie blue. Gels: 1) Control membranes; 2) Control cytoskeletons; 3) Cytoskeletons crosslinked for 0 min; 4) Cytoskeletons crosslinked for 10 min; 5) Cytoskeletons crosslinked for 20 min; 6) Cytoskeletons crosslinked for 30 min. Arrows at the top of the photographs indicate crosslinked products.

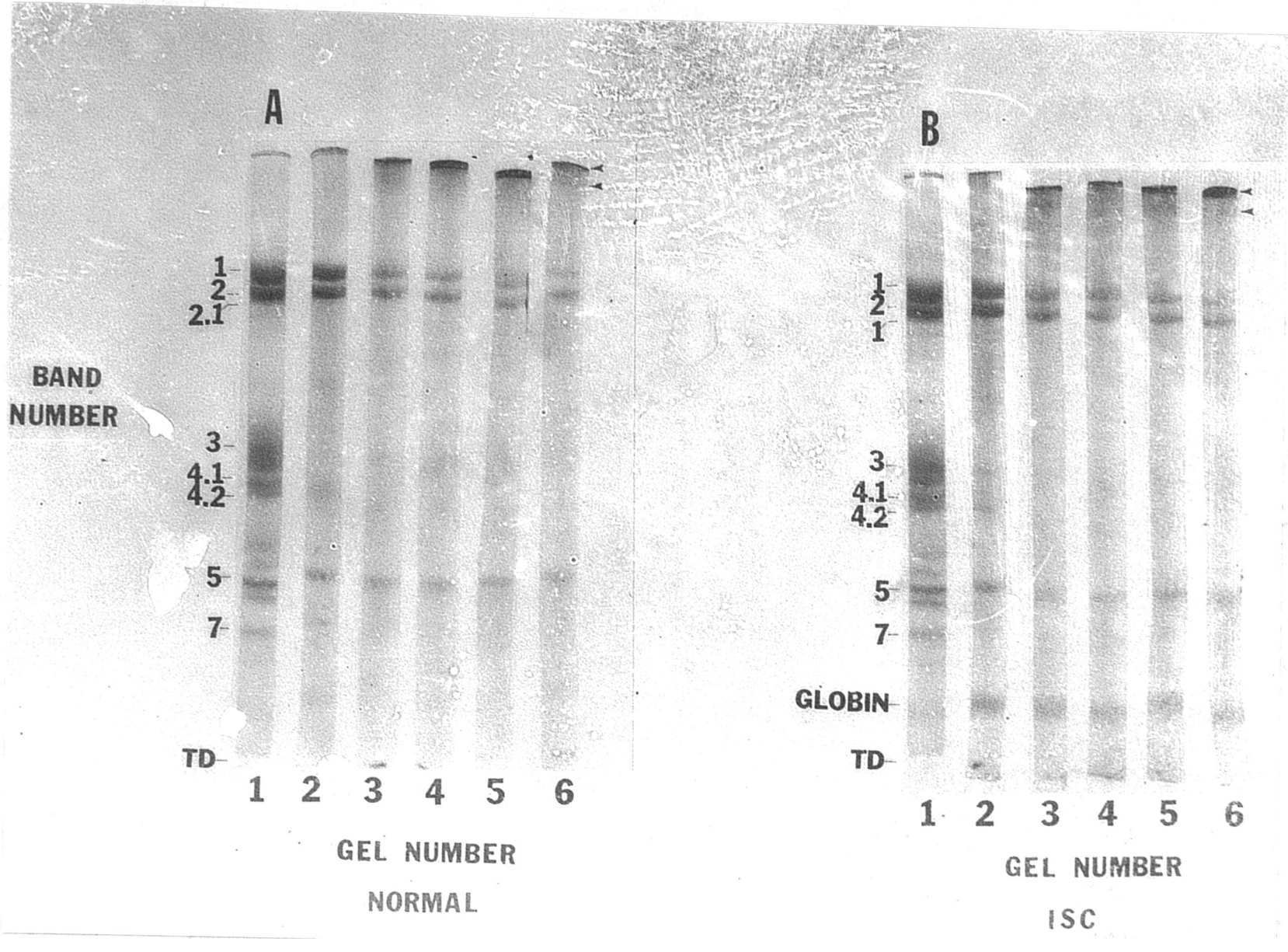
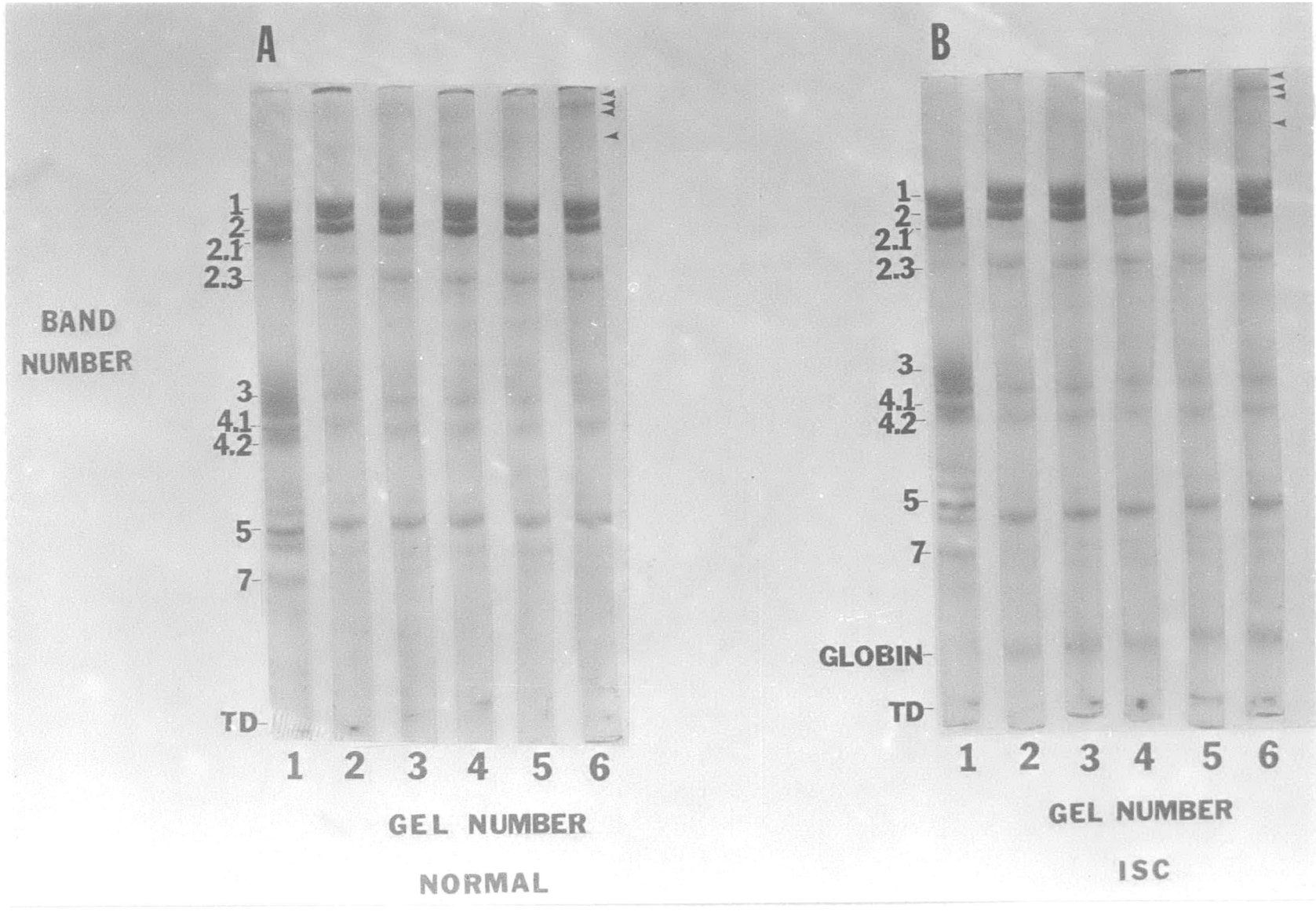

A
BAND NUMBER
1
2
2.1
3
4.1
4.2
5
7
TD
1 2 3 4 5 6
GEL NUMBER
NORMAL
B
1
2
1
3
4.1
4.2
5
7
GLOBIN
TD
1 2 3 4 5 6
GEL NUMBER
ISC

Figure 23. Crosslinking of cytoskeletons from (A) Normal and (B) ISC's with 60mM Dimethyladipimidate (DMA). Electrophoresis was carried out in 4% Fairbanks gels and the gels stained with Coomassie blue. Gels: 1) Control membranes; 2) Control cytoskeletons; 3) Cytoskeletons crosslinked for 0 min; 4) Cytoskeletons crosslinked for 10 min; 5) Cytoskeletons crosslinked for 20 min; 6) Cytoskeletons crosslinked for 30 min. Arrows at the top of the photographs indicate crosslinked products.


A
BAND NUMBER
1
2
2.1
2.3
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4.1
4.2
5
7
TD
1 2 3 4 5 6
GEL NUMBER
NORMAL
B
1
2
2.1
2.3
3
4.1
4.2
5
7
GLOBIN
TD
1 2 3 4 5 6
GEL NUMBER
ISC

apparent. The crosslinked products in both ISC and normal cytoskeletal preparations appeared to be the same.

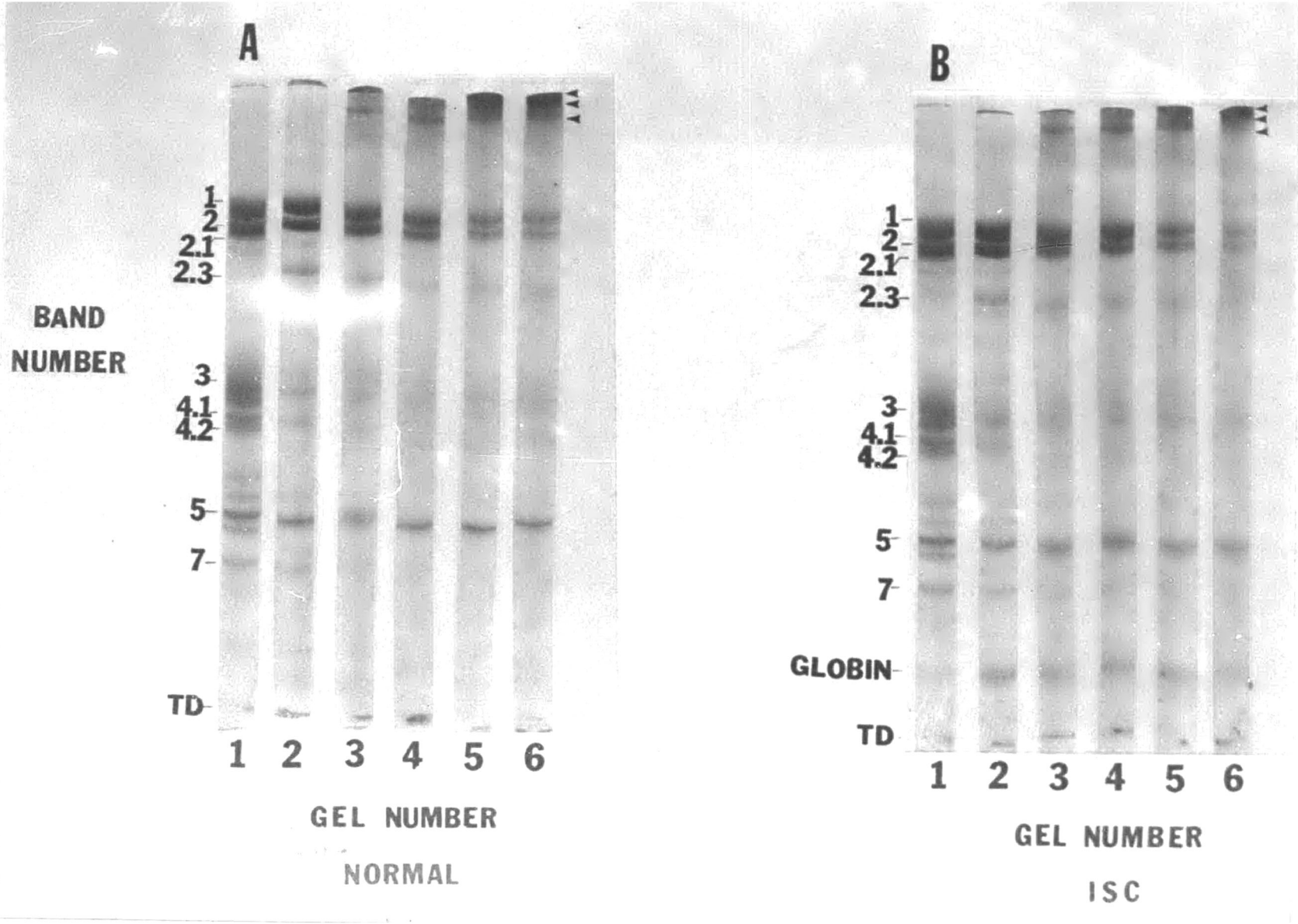
Dimethylsuberimidate (DMS)

DMS, another imidoester, is very similar to DMA, but the reactive groups are 11 Å apart. DMS was far more effective in crosslinking cytoskeletal preparations than DMA (Fig. 24). Conceivably this is due to the fact that it is longer.

Bands 1, 2, and 4.1 were reduced with increasing time of crosslinking, suggesting that they were the major components of the crosslinked complexes. In contrast, the intensity of band 5 seemed to be relatively constant as a function of time of crosslinking.

As with the other crosslinking reagents, no difference was observed between ISC and normal cytoskeletons.

Figure 24. Crosslinking of cytoskeletons from (A) Normal and (B) ISC's with 80mM Dimethylsuberimidate (DMS). Electrophoresis was carried out on 4% Fairbanks gels, and the gels stained with Coomassie blue. Gels: 1) Control membranes; 2) Control cytoskeletons; 3) Cytoskeletons crosslinked for 10 min; 5) Cytoskeletons crosslinked for 20 min; 6) Cytoskeletons crosslinked for 30 min. Arrows at the top of the photographs indicate crosslinked products.


A
BAND NUMBER
1
2
2.1
2.3
3
4.1
4.2
5
7
TD
1 2 3 4 5 6
GEL NUMBER
NORMAL
B
1
2
2.1
2.3
3
4.1
4.2
5
7
GLOBIN
TD
1 2 3 4 5 6
GEL NUMBER
ISC

CHAPTER V

DISCUSSION

Studies by Lux et al. (1976) indicate that the defect(s) responsible for abnormal cell shape of ISC's resides within the cytoskeletal proteins. The primary goal of this research was to define the defect(s) in the ISC cytoskeleton. The long range goal of this project is to determine whether this defect is responsible for the abnormal cell shape.

ISC's seem to play an important role in the expression of sickle cell anemia. Infarction in the spleen (Sergeant, 1970) and conjunctiva (Sergeant et al., 1972), as well as the degree of anemia (Sergeant et al., 1969), seem to be due to the presence of ISC's. Although no good correlations have been established between the vaso-occlusive ("pain") crisis and the presence of ISC's (Hebbel et al., 1979; Sergeant et al., 1969; Steinberg et al., 1977) experimental results suggest that ISC's may initiate this symptom of the disease (Klug et al., 1974).

In theory, there are two alterations of red cells that would restrict their passage through capillaries: (1) reduced deformability and (2) increased adhesivity. Several lines of evidence suggest that both of these alterations are characteristics of ISC's. ISC's are less filterable than are normal cells (or non-ISC's) (Chien et al., 1970) and this appears to be due to cellular dehydration (Shohet, 1979) and also increased rigidity of the membrane (LaCelle and Kirkpatrick, 1975). It has been shown recently that ISC's adhere more avidly to endothelial cells in culture than do non-ISC's and that non-ISC's bind more tightly than normal cells. This may be due to an abnormal distribution of negatively charged residues on the surface of the cell (Hebbel et al., 1979). Although these latter investigators did not observe a correlation between microvascular occlusions and ISC's, they did note a correlation

between adherence to endothelial cells and the frequency of vaso-occlusive crisis. While these data suggest that non-ISC's may also initiate vaso-occlusions, it seems reasonable that ISC's play a disproportionate role since ISC's are the most adhesive S/S erythrocytes. Possibly the correlation between ISC's and microvascular occlusions is masked due to the fact that many variables affect this symptom of the disease.

There is reason to believe that a single defect in the cytoskeleton may be responsible for abnormal ISC shape, rigidity and increased adhesivity. Since it has been established that the ability to undergo changes in cell shape, i.e., the ability to be deformed, is due to the cytoskeleton (Lange et al., 1982), this implies that the irreversible deformation of the cytoskeleton should lead to increased membrane rigidity. The hypothesis that the shape defect is also a possible cause of increased adhesivity is supported by the observation that the specific aggregation of spectrin causes changes on the external surface of the cell, and specifically, the aggregation of negatively charged sites (Nicholson et al., 1971).

In order to detect defects in ISC cytoskeletons, they have been directly examined with the electron microscope and by SDS-gel electrophoresis using the Fairbanks system. No differences in the fine structure or protein composition were observed (Lux et al., 1976). When ISC's are incubated in the presence of radioactive phosphorous (^{32}P), the β-chain of spectrin is phosphorylated less readily than the corresponding polypeptide in normal cells (Dzandu and Johnson, 1980), and it has been shown that a spectrin-rich polymer is formed more readily in ISC's than normal cells upon ATP depletion. Although this latter change was not detectable with either the crosslinking agents glutaraldehyde or o-phenanthroline, both studies involving whole cells suggest that spectrin has an abnormal arrangement in the ISC cytoskeleton. However, as previously mentioned, it has not been established

whether the spectrin abnormalities detected in ISC's are a cause of its abnormal shape.

While the above alterations have been detected in ISC cytoskeletons, it is possible that other alterations exist. In recent years, a number of improvements have been made in techniques to characterize proteins by electrophoresis, so that we are now capable of achieving greater resolution than when the studies of Lux et al. (1976) were performed. For example, two-dimensional electrophoresis is capable of separating proteins which differ by only a single charge (O'Farrell, 1975). Moreover, since the studies of Palek and Liu (1979), a variety of new crosslinking agents have been synthesized. Because of the resolving capacity of these new techniques and since the major objective of this work was to detect differences in ISC cytoskeleton proteins, the methods used in this study were thought to be the most suitable ones.

Generally, the ISC cytoskeleton has been re-examined using a battery of approaches which, when taken together, represents the most precise approach used thus far to detect alterations in ISC cytoskeletons. Through use of Dextran-gradients, a purer fraction of ISC's was obtained than previously isolated for studies of cytoskeletal proteins. Analyses of these proteins on SDS-Laemmli-gradient gels are shown in this study to be more resolving than electrophoretic systems previously used. The use of several stains, which have only recently been used to detect proteins in gels, have permitted detection of minor proteins. The data obtained in this study lead me to conclude that the cytoskeleton may be more complex than previously thought (Lux, 1979). Isoelectric focusing and two-dimensional electrophoresis have not been used previously to analyze ISC cytoskeletal proteins. It was thought, among the techniques selected for use in this investigation, that two-dimensional electrophoresis had the greatest potential for revealing an alteration in ISC cytoskeletal proteins.

It is a reasonable assumption that the arrangement of proteins in ISC's is altered, based on the fact that the ISC cytoskeleton has a different shape. Crosslinking reagents are the best probes of supramolecular structure and have been widely used to study the quaternary structure of proteins (Davies and Stark, 1970). A variety of these reagents differing in mechanism of action and distance between reactive groups were employed in order to maximize the possibility of detecting differences in protein organization.

Although a variety of modern techniques were used to detect a difference in ISC cytoskeletons, I have been unable to detect a difference in either the composition or organization of proteins. There are several reasons why a defect may not have been detected. First, it is possible that the actual concentration of defective cytoskeletons in the bottom fraction was much less than indicated by the ISC count (71%). If this was the case, the defect in the cytoskeleton may have been masked by normal proteins. A second possible explanation is that the electrophoretic and crosslinking techniques were not sufficiently resolving to detect a difference.

Lux et al. (1976) have shown that only 56% of the cells which maintain the sickled shape after oxygenation, and are thus defined as ISC's, yield sickled-shaped membranes. He has suggested that those cells yielding normally-shaped membranes are actually incompletely oxygenated non-ISC's. If the percentage of oxygenated sickled cells in the bottom fraction studied here yielding normally-shaped ghosts was comparable to that in the experiments of Lux et al., then the percentage of defective cytoskeletons may be as low as 35% rather than 71% suggested by the ISC count.

There were several problems encountered in performing the electrophoretic and crosslinking experiments which may have limited their resolving power. The

large number of prominent spots seen in two-dimensional gels on which cytoskeleton preparations were electrophoresed is possibly artifactual because the conventional view is that there are a limited number of polypeptides in the cytoskeleton (Lux, 1979). The observation that multiple spots appear on several horizontal planes in the two-dimensional gels is evidence for charge modification (O'Farrell, 1975), and the fact that a number of polypeptides differing in molecular weight appear focused the same pI near the cathode end of the gel is evidence for aggregation of proteins (O'Farrell, 1975). The fact that crosslinked intermediates were not observed, coupled with the inability to resolve the various crosslinked complexes near the origin of the gel, reduced the effectiveness of this technique.

In these studies, no alteration was detected in the ISC cytoskeleton, however, several observations add to our understanding of the erythrocyte cytoskeleton. The five to six minor Gelcode-positive polypeptides of lower molecular weight than band 7, and the approximately six negatively-stained polypeptides in the 4.5 region detected here, have not previously been reported. These findings suggest that the cytoskeleton contains, in addition to five well-known polypeptides constituents, 1, 2, 4.1, 5 and 7, a number of minor polypeptide components.

It is also of considerable interest that band 5 is relatively resistant to being crosslinked. Since a variety of crosslinking agents were used in this study, this observation may indicate that band 5 (actin) has an unusual organization in the cytoskeleton. Current models of the cytoskeleton indicate that bands 5 and 4.1 join spectrin molecules in the network. Since both spectrin and band 4.1 are crosslinked in the experiments reported here, it is puzzling that band 5 is not. Perhaps it is arranged in the cytoskeleton in such a way that it is unaccessible to the crosslinking reagents.

There are several technical improvements that could be made in order to

increase the possibility of detecting an alteration in the ISC cytoskeleton. Cytoskeletons have been viewed with the electron microscope (Lux et al., 1976; Yu et al., 1973), and this technique was essential in order to determine whether ISC cytoskeletons in my experiments were sickled-shaped. Several unsuccessful efforts were made in the course of this work to view cytoskeletons with the electron microscope; however, a recent work suggests that this is relatively easily done by extracting cells with Triton after attaching them to polylysine - coated grids (Sheetz and Sawyer, 1978).

Assuming that some ISC's will yield normally-shaped cytoskeletons, it would be useful to develop a method of fractionating cytoskeletons with sickled-shapes. The transmission electron micrographs of Lux et al. (1976) suggest that the proteins in the sickled-shaped ISC cytoskeleton are more dense than in normally-shaped cytoskeletons. Thus, it may be possible to separate the two types of cytoskeletons by density gradient centrifugation.

The two-dimensional electrophoretic analyses carried out here were not as resolving as theoretically possible because of the apparent artifactual charge modification and aggregation of proteins. There are several precautions that might be utilized in future studies to minimize artifact (O'Farrell, 1975).

There are several improvements that can be made in the crosslinking analytic procedure. In order for the very high molecular weight complexes to penetrate into the gel so that they may be analyzed, the gel pore size can be increased by using a lower concentration of acrylamide. In the studies described in this thesis, 4% acrylamide gels were used. Peacock and Dingman (1968) has described a procedure for making high porosity gels, using 1% acrylamide fortified with agarose.

The fact that intermediate crosslinked products were apparently not detected in the gel further limited my ability to detect differences between ISC and normal

cytoskeletal proteins. Further examination of various crosslinking lead to definition of crosslinking conditions which yield intermediate crosslinked complexes.

Finally, a new method for analyzing cytoskeletal proteins which seems capable of extremely high resolution, has recently been developed by Marchesi's group (1983). The method involves fractionating proteolytic fragments of purfied proteins by two-dimensional electrophoresis followed by isolation, subsequent proteolysis, and finger printing (Ingram, 1956) of each molecular fragment obtained after the first two-dimensional electrophoresis. This technique has been used to analyze the spectrin molecule and, as an indication of its high resolving power, spectrin from normal erythrocytes from blacks has been shown to differ in one peptide from spectrin obtained from normal erythrocytes from whites. Analysis of each cytoskeleton protein in the ISC with this method may be the most resolving approach to detect an ISC defect.

The most fundamental difference between the ISC and normal cytoskeleton thus far reported is a reduction in the phosphorylation of band 2 in the ISC (Dzandu and Johnson, 1980). Since band 2 is phosphorylated at four to five sites near its carboxyl terminus in normal spectrin (Marchesi, 1983), it would be of interest to determine which sites in ISC spectrin do not carry phosphate groups. Subsequent studies could aim at establishing the mechanism by which the reduction in phosphorylation occurs, and whether there is a causal relationship between this defect and the abnormal cell shape.

The recent work of Hebbel et al. (1979) suggests that increased adhesivity of ISC's may be more important in causing vaso-occlusions than the reduced deformability. If this is true, it may be profitable to attempt to better characterize the molecular nature of the membrane surface defect. Of course, the next step would be to establish the mechanism responsible for the molecular alteration in the

surface. Since it has been shown that spectrin is connected to all external proteins and carbohydrates via transmembrane proteins (Nicholson et al., 1971), it would be of great interest to determine whether a defect in the cytoskeleton that is responsible for the abnormal shape is also the cause of the alteration in the membrane surface.

CHAPTER VI

SUMMARY

Several conclusions were reached based on the data obtained in this study:

1. No difference can be detected between the protein composition of ISC and normal erythrocyte cytoskeletons when analyzed on 5-15% gradient gels utilizing the Laemmli buffer system, after staining with Coomassie blue, Silver, or Gelcode.

2. No difference can be detected between the protein composition of ISC and normal cytoskeletons when analyzed by Isoelectric Focusing and stained conventionally.

3. No difference was detected in the protein composition of ISC and normal erythrocyte cytoskeletons when analyzed by two-dimensional electrophoresis; however, since artifactual spots were observed, it is not certain that this technique may not yet reveal a difference between the two types of cytoskeletons.

4. No difference was detected in the protein organization of ISC and normal erythrocyte cytoskeletons when analyzed with a battery of crosslinking reagents.

Although it is clear that a defect is present in the ISC cytoskeleton, it could not be detected using the above techniques. However, in the course of this study, several observations were made which may lead to new models of the cytoskeleton. Several minor proteins were detected with Silver and Gelcode which had not been reported, and actin, in contrast to other cytoskeletal proteins, was refractory to crosslinking.

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